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The Role of Intestinal Inflammation on the Gut-Liver Axis

PhD student

Noemi Fiaschini

Supervisor

Prof. **Salvatore Cucchiara**

Co-supervisor

Prof. **Laura Stronati**

Co-supervisor (ENEA)

Dr. **Anna Negroni**

Summary of the whole project

Background:

The gut and the liver are anatomically related by portal circulation, and their functional unit realizes the gut-liver axis (GLA) with the integrity of intestinal barrier crucial for the maintenance of liver homeostasis.

The GLA connects the liver with the intestine via bile acid metabolism. Bile acids (BAs) are amphipathic steroid acids, synthesized from cholesterol in the liver, that regulate lipid, glucose and energy metabolism. Moreover, a specific role of BAs as immunomodulators is emerging.

The regulatory functions of BAs are predominantly mediated by the bile receptors, such as the nuclear receptors farnesoid-X-receptor (FXR) and pregnane-X-receptor (PXR) as well as the membrane G-protein-coupled-receptor TGR5.

Recent findings suggest that the occurrence of gut inflammation, featured by altered epithelial and vascular permeability that causes the major translocation of bacterial antigens, may affect the healthy liver as well as worsen the severity of liver diseases, in particular of the non-alcholic fatty liver disease (NAFLD) and its progressive form, the non-alcoholic steatohepatitis (NASH).

Aims:

Accordingly, the whole purpose of this project is to assess the impact of gut inflammation on liver health and disease

For this purpose, in a first part of the thesis we aimed to:

1. Investigate *in vitro* and *in vivo* the possibility that gut inflammation affects the healthy liver by altering BA receptors FXR, PXR and TGR5 and increasing the production of inflammatory and oxidative stress molecules;
2. analyze *ex vivo* the expression pattern of BA receptors and the apical sodium-dependent bile acid transporter (ASBT) in the inflamed colonic tissues of a group of pediatric patients with inflammatory bowel disease (IBD) and age-matched controls.

In a second part of the thesis, we aimed to:

1. develop an animal model of hepatic steatosis, displaying the disease both in its early (NAFLD) and late (NASH) phase, in which an important intestinal inflammation was also induced;
2. use this model to assess that gut inflammation significantly contributes to the progression of the liver disease (from NAFLD to NASH) by altering BA receptor expression and increasing inflammatory (IL-6, TNFα, NLRP3, TLR4, MCP-1, HMGB1) as well as fibrotic (TGF-β, α-SMA) mediator expression;
3. evaluate the potential of the anti-inflammatory molecule, the dipotassium glycyrrhizate (DPG), to improve the liver disease by reducing gut inflammation.

Results:

Results and conclusions of the first part of experimentation are reported in the original paper published in Journal of Pediatric Gastroenterology and Nutrition (Negroni A, Fiaschini N, Palone F, Vitali R, Colantoni E, Laudadio I, Oliva S, Aloi M, Cucchiara S, Stronati L. "Intestinal inflammation alters the expression of hepatic bile acid receptors causing liver impairment" J Pediatr Gastroenterol Nutr. 2020 Aug;71(2):189-196) that has been attached to the thesis.

Results of the second part of experimentation are fully described in the thesis. We have developed an animal model with intestinal inflammation and liver steatosis/steatohepatitis by treating C57BL/6J mice with dextran sodium sulphate (DSS) to induce colitis and high fat diet (HFD) with high glucose/fructose for different times to induce NAFLD/NASH. Mice with NAFLD/NASH without colitis served as control group. A further group of NAFLD/NASH-DSS-mice were also treated with DPG.

Results show that gut inflammation, assessed by the release of the alarmin HMGB1 in the stools, and consequent altered intestinal epithelial and vascular permeability, confirmed by a reduced expression of the tight junction protein zonulin-1 (ZO-1) and an increased level of the endothelial cell-specific protein plasmalemma vesicle-associated protein 1 (PV1), correlate with altered BAs receptor expression (TGR5 and PXR), increase of inflammatory marker expression (IL-6, TNFα, NLRP3, TLR4, MCP-1, HMGB1) and inflammatory infiltrate in the steatotic liver of NASH-DSS mice. Moreover, the latter showed a significant rise of collagen fiber deposition and increased fibrotic marker (α-

SMA and TGF-β) expression as compared to DSS-mice. The administration of DPG to DSS-NASH mice significantly reduced these effects.

Conclusions:

These data confirm our hypothesis that the presence of gut inflammation causes liver injury and accelerates fibrosis in a steatotic liver, contributing to the progression of NAFLD towards NASH. We also suggest that reducing gut inflammation by using DPG could represent an interesting novel strategy for the management of the hepatic disease.

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List of abbreviations

AB: Alcian blue staining. **ALT:** alanine aminotransferase. **AMPs:** antimicrobial peptides. **ASBT:** the apical sodium-dependent bile acid transporter. **AST:** aspartate aminotransferase. **BAs:** bile acids. **BMI:** body mass index. **CA:** cholic acid. **CDCA:** chenodeoxycholic acid. **CYPs:** 17 cytochrome P450 enzymes. **DAI:** disease activity index. **DCA:** deoxycholic acid. **DG:** Diammonium glycyrrhizinate. **DPG:** Dipotassium glycyrrhizate. **DSS:** dextran sodium sulphate. **FGF19:** Fibroblast growth factor 19. **FISH:** fluorescence in situ hybridization **FMT:** fecal microbiota transplantation. **FXR:** farnesoid X receptor. **FXRE:** FXR response element. **GI:** gastrointestinal. **GLA:** gut-liver axis. **GLP-1:** glucagon-like peptide 1. **GM:** gut microbiota. **GVB:** gut-vascular barrier. **H&E:** hematoxylin and eosin. **HFD:** high fat diet. **HMGB1:** protein High Mobility Group Box 1. **HTN:** hypertension. **IBD:** inflammatory bowel disease. **IBS:** inflammatory bowel syndrome. **IHC:** Immunohistochemical staining. **iEC:** intestinal epithelial cell. **IgA:** immunoglobulin A. **IR:** insulin resistance. **LCA:** lithocholic acid. **LDL:** Low Density Lipoproteins. **LPS:** lipopolysaccharide. **MAPK:** the mitogen-activated protein kinase. **MetS:** metabolic syndrome. **MLNs:** mesenteric lymph nodes. **NAFL:** non-alcoholic fatty liver. **NAFLD:** Non-alcoholic fatty liver disease. **NAS:** NAFLD activity score **NASH:** non-alcoholic steatohepatitis. **NF-kB:** nuclear factor κB. **NRs:** Nuclear receptors. **OCA:** obeticholic acid. **PAMP:** pathogen associated molecular patterns. **PAS:** Periodic Acid – Schiff's staining. **PPAR:** peroxisome proliferator-activated receptor. **PUFA:** Polyunsaturated fatty acid. **PRRs:** Pattern recognition receptors. **PXR:** pregnane X receptor. **PXRE:** PXR responsive element. **PV1:** protein plasmalemma vesicle-associated protein 1. **SCFAs:** short-chain fatty acids. **TGR5:** G protein-coupled bile acid receptor. **TLCA:** taurolithocholic acid. **TLRs:** Toll-like receptors. **TJ:** tight junction. **TZDs:** thiazolidinediones. **T2D:** type 2 diabetes. **UDCA:** ursodeoxycholic acid. **ZO1:** protein zonulin-1.

Intestinal Inflammation Alters the Expression of Hepatic Bile Acid Receptors Causing Liver Impairment

*Anna Negroni, ††Noemi Fiaschini, †Francesca Palone, *Roberta Vitali, †Eleonora Colantoni, §Ilaria Laudadio, †Salvatore Oliva, †Marina Aloi, †Salvatore Cucchiara, and §Laura Stronati

ABSTRACT

Objectives: The gut-liver axis has been recently investigated in depth in relation to intestinal and hepatic diseases. Key actors are bile acid (BA) receptors, as farnesoid-X-receptor (FXR), pregnane-X-receptor (PXR), and G-protein-coupled-receptor (GPCR; TGR5), that control a broad range of metabolic processes as well as inflammation and fibrosis. The present study aims to investigate the impact of intestinal inflammation on liver health with a focus on FXR, PXR, and TGR5 expression. The strategy to improve liver health by reducing gut inflammation is also considered. Modulation of BA receptors in the inflamed colonic tissues of inflammatory bowel disease (IBD) pediatric patients is analyzed.

Methods: A dextran sodium sulphate (DSS) colitis animal model was built. Co-cultures with Caco2 and HepG2 cell lines were set up. Modulation of BA receptors in biopsies of IBD pediatric patients was assessed by real-time PCR and immunohistochemistry.

Results: Histology showed inflammatory cell infiltration in the liver of DSS mice, where FXR and PXR were significantly decreased and oxidative stress was increased. Exposure of Caco2 to inflammatory stimuli resulted in the reduction of BA receptor expression in HepG2. Caco2 treatment with dipotassium glycyrrhizate (DPG) reduced these effects on liver cells. Inflamed colon of patients showed altered FXR, PXR, and TGR5 expression.

Conclusions: This study strongly suggests that gut inflammation affects hepatic cells by altering BA receptor levels as well as increasing the production of pro-inflammatory cytokines and oxidative stress. Hence, reducing gut inflammation is needed not only to improve the intestinal disease but also to protect the liver.

Key Words: bile acid receptors, gut, inflammation, inflammatory bowel disease, liver

(*JPGN* 2020;71: 189–196)

What Is Known

- Gut-liver axis connects liver and intestine via bile acid metabolism.
- Bile acid receptors, regulating lipid and glucose metabolism, are currently known to be also involved in immune and inflammatory response.

What Is New

- Gut inflammation alters farnesoid-X-receptor, pregnane-X-receptor, and G-protein-coupled receptor (TGR5) levels and increases the production of pro-inflammatory cytokines and oxidative stress in the liver.
- The amelioration of intestinal inflammation causes liver improvement as shown by the bile acid receptor level restitution.
- Children with inflammatory bowel disease show altered farnesoid-X-receptor, pregnane-X-receptor, and TGR5 expression in the inflamed colon.

The gut and the liver are anatomically connected by portal circulation, and their functional unit realizes the gut-liver axis (GLA) with the integrity of intestinal barrier crucial for the maintenance of liver homeostasis (1–3). In this mutual relationship, the liver acts as a second firewall towards potentially harmful substances translocated from the gut, and in turn is implicated in the regulation of the mucosal barrier (4).

The GLA connects the liver with the intestine via bile acid metabolism (5–6). Bile acids (BAs) are amphipathic steroid acids, synthesized from cholesterol in the liver, that regulate lipid, glucose, and energy metabolism (7–9). Moreover, a specific role of BAs as immunomodulators is emerging (5,10–14).

The regulatory functions of BAs are predominantly mediated by the bile receptors, such as the nuclear receptors farnesoid-X-receptor (FXR) and pregnane-X-receptor (PXR) as well as the membrane G-protein-coupled receptor (GPCRs; TGR5) (15,16).

Nuclear receptors are ligand-dependent transcription factors that regulate a variety of physiological processes, such as homeostasis, reproduction, development, inflammation, and metabolism, by inducing the transcription of target genes (15). FXR is highly expressed in the liver and gut and affects lipid and glucose metabolism (17). Similarly, PXR is involved in the regulation of xenobiotic metabolism (18), although, recent evidences outline its role also in inflammatory response, cell proliferation, and migration (19).

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From the *Division of Health Protection Technologies, ENEA, the †Maternal Infantile and Urological Sciences Department, the ‡Innovative Biomedical Technologies in Clinical Medicine at Sapienza, and the §Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy.

Address correspondence and reprint requests to Anna Negroni, PhD, Division of Health Protection Technologies, ENEA: Italian National Agency for New Technologies, Energy and Sustainable Economic Development, Via Anguillarese 301, 00123 Rome, Italy (e-mail: anna.negroni@enea.it).

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TGR5 is a member of GPCRs, ubiquitously expressed in diverse tissues, including endocrine organs, muscle, adipose tissue, immune cells, and intestinal tract (20). Recent literature has extended its functions to more than metabolic regulation, which include inflammatory response, cancer, and liver regeneration (20–22). Upon binding with lithocholic acids (LCA) and taurolithocholic acid (TLCA), it is activated to transduce signal transduction into the nucleus and control diverse gene expression (23).

The enterohepatic circulation of BAs is governed by specific transporters expressed in the liver and the intestine and plays a critical role in the digestion of fats and oils. During this process, the majority of the BAs secreted from the liver is reabsorbed in intestinal epithelial cells via the apical sodium-dependent bile acid transporter (ASBT/SLC10A2) and then transported into the portal vein (24).

In this setting, BAs and their receptors are attractive candidates for therapeutic development in chronic diseases, such as type 2 diabetes, hypertriglyceridemia, obesity, nonalcoholic steatohepatitis, inflammatory bowel disease (IBD), and sclerosing cholangitis (25–29).

To deeper investigate the strict relationship between the liver and the gut, the present study aims to assess in vivo and in vitro, the impact of gut inflammation on liver health by focusing on the modulation of BA receptors FXR, PXR, and TGR5. The strategy to improve liver health by reducing gut inflammation is also considered. Moreover, given the role of BA receptors in controlling intestinal mucosal immunity and inflammation, a secondary goal is to analyze the expression pattern of FXR, PXR, and TGR5 BA receptors as well as the ASBT transporter in the inflamed colonic tissues of pediatric patients with IBD and in controls.

METHODS

Ethical Statement

This work has been approved by the Ethic Committee of the Umberto I Hospital, Sapienza University of Rome, Italy. All parents of patients entered into the study provided written informed consent. Experimental procedures on mice were previously approved by the Ministry of Health.

Cell Lines

The human colon adenocarcinoma cell line Caco2, the hepatic adenocarcinoma cell line HepG2, and the murine macrophage-like cell line RAW264.7 were purchased from ATCC (Rockville, MD). Inflammation was induced by cytomix, a combination of TNF-alpha (10 ng/mL; Sigma, St. Louis, MO) and Interferon-gamma (250 ng/mL).

Co-culture System

Cells will be seeded for differentiation on ThinCert cell culture polyethylene terephthalate (PET) capillary pore membranes (0.4 μm pore diameter; Greiner Bio-One International GmbH) and maintained in complete medium supplemented with 10% FBS in both apical (AP) and basolateral (BL) compartments. For co-culture experiments, Caco2 cells differentiated on filter inserts will be transferred to culture plates containing confluent HepG2 cells. Treatment with cytomix will be added to the AP compartment of Caco2 cells and the culture plates will be incubated at 37 °C for 24 and 48 hours. Alternatively, Caco2 cells were infected with LF82 strain at a MOI of 10 : 1 for 3 hours. Then medium with bacteria were removed and replaced with new medium for additional 3 hours.

Trans-epithelial Electric Resistance Assay

Caco2 cells were grown on polyethylene terephthalate membrane inserts, pore size 0.4 mm (Falcon, Becton Dickinson, Franklin Lakes, NJ). TEER values were measured using a Millicell-ERS voltohmmeter (Millipore, Billerica, MA) according to the Manufacturer's instructions.

Animal

C57BL/6 female mice (8–9 weeks of age) were purchased from the animal housing unit of Harlan Laboratories, SRL. Induction of colitis was performed through administration of dextran sodium sulphate (DSS, molecular mass, 36,000–50,000 Da, MP Biomedicals, Santa Ana, CA), 3% dissolved in autoclaved drinking water, for 7 days. Clinical score (CS) was assessed according to the criteria of Maxwell et al (30). On the seventh day, animals were euthanized. Distal colonic, and liver specimens were fixed immediately in a 10% (w/v) formalin solution for histological analysis and frozen in liquid nitrogen or for molecular analyses.

Patients

Ten patients with Crohn disease (CD) (median age: 13.0 years; range: 6–17 years), 10 with ulcerative colitis (UC) (median age: 12.9 years; range: 7–17 years), and 10 controls (median age: 11 years; range: 5–17 years), referred to the Maternal Infantile Department and Urological Sciences, at the Sapienza University of Rome and needed an ileocolonoscopy to reassess the intestinal disease, were included in this study. All selected patients had an established diagnosis of IBD and were in active phase of disease even under therapy. Patients were under treatment with immunomodulators (azathioprine or methotrexate), mesalamine, or oral corticosteroids at low doses. Activity in CD and UC was measured, respectively, by the PCDAI (Pediatric Crohn's Disease Activity Index) (31) and the PUCAI (Pediatric Ulcerative Colitis Activity Index) score (32). The intestinal inflammation was assessed at endoscopy by using the SES-CD score (33) and the endoscopic Mayo subscore (34) in CD and UC patients, respectively. Children with incapacitating functional gastrointestinal disorders requiring extensive investigation, having normal endoscopy and histology, served as controls.

Biopsy Treatment

Colonic mucosal specimens were immediately snap frozen in liquid nitrogen for RNA analysis or fixed immediately in a 10% (w/v) formalin solution for histology and immunohistochemistry.

Real-time Polymerase Chain Reaction

Total RNA was isolated from cells and biopsies using the RNeasy kit (QiaGen, Hilden, Germany). Total RNA (1 μg) was reverse-transcribed to cDNA by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR amplification was done with an ABI PRISM 7300 Sequence Detection System using the SYBR Green kit (Applied Biosystems). The quantity of each mRNA to a reference gene was calculated by the $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry

Sections (4 μm) of paraffin-embedded intestinal inflamed tissues were prepared following standard protocol. Anti-FXR and anti-TGR5 (Abcam, Cambridge, UK) antibody were used. Finally, sections were stained with hematoxylin and eosin.

Statistics

A minimum of 5 mice per group were included in all experiments. Statistical analysis for significance was determined using the GraphPad InStat software. The Kolmogorov-Smirnov test showed significant departures from the normal distribution for some of the analyzed parameters. Therefore, comparison of the group was performed by the Mann-Whitney *U* test. For in vitro experiments, the Student *t*-test was used. Experiments were repeated 3 times. Data were presented as mean ± SD. Differences were noted as significant $^{*}P < 0.05$, and $^{**}P < 0.01$.

RESULTS

Farnesoid-X-receptor, Pregnane-X-receptor, and TGR5 Levels are Significantly Altered in the Inflamed Colon as well as in the Liver of Mice With Dextran Sodium Sulphate-induced Colitis

We used the 3% DSS-treated mice as a model of severe colitis. The occurrence of gut inflammation was confirmed by histology, which showed the loss of crypt structure, extensive inflammatory cell infiltration and thickening of the mucosa with abundant edema (Fig. 1A). Interestingly, increased inflammatory cells were also detected in the hepatic tissue of DSS mice as compared with controls (Fig. 1B). Moreover, as expected, inflamed colonic tissues showed increased levels of pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β ($P < 0.01$); however, IL-6, and IL-1β were strongly increased also in the liver ($P < 0.01$) (Fig. 1C).

FXR and PXR mRNA expression was significantly decreased in the inflamed colon of DSS-treated mice ($P < 0.05$), whereas an increased expression of TGR5 was found ($P < 0.05$) (Fig. 1D). This finding could be explained by recent data reporting the occurrence of higher concentration of TGR5 in macrophages that are highly involved in the tissue inflammation processes (35). To deeper investigate this point, we performed immunohistochemistry on mucosal colonic samples of mice and found that whereas FXR was mainly localized in the nuclei of enterocytes, TGR5 was largely confined to phagocytes (Fig. 1E). Then, we used the murine macrophages RAW 264.7 to confirm in vitro that inflammation enhances the levels of TGR5 receptor. RAW 264.7 cells were exposed to a mix of pro-inflammatory cytokines (cytomix: TNFα+INFγ) for 6 or 24 hours. Results showed a significant increase of TGR5 ($P < 0.01$), whereas FXR and PXR were very poorly expressed (data not shown). Accordingly, intestinal epithelial cells Caco2 and liver cells HepG2 exposed to the same inflammatory agents showed a decrease of all BA receptors, including TGR5 ($P < 0.05$) (Fig. 1F).

Remarkably, we observed a significant decrease of FXR and PXR in the liver of mice ($P < 0.05$) and a substantially unchanged level of TGR5 as well (Fig. 1G). As it is increasingly recognized that FXR has antioxidant activities, in order to better characterize the liver impairment induced by intestinal inflammation, we also analyzed in hepatic tissues, the gene expression of nitric oxide synthase (iNOS) and cyclooxygenase (COX2), that have been shown to play pivotal roles in the development of inflammatory diseases, including cancer. We found that both enzymes were importantly up-regulated ($P < 0.01$) in the liver of DSS-treated mice (Fig. 1H).

Co-cultures of Intestinal and Hepatic Cells Show that Gut Inflammation Directly Affects Liver Cells by Altering Bile Acid Receptor Expression

We set up a co-culture system with differentiated and oriented Caco2 (above) and HepG2 (below) (Fig. 2A). The full confluence of Caco2 to form a whole intestinal barrier was proven by the transepithelial electric resistance (TEER) assay (Fig. 2B). Intriguingly, we observed upon exposure of only Caco2 to the cytomix (24 and 48 hours) inflammation in HepG2 cells occurred, as shown by the increase of IL-8 and IL1-β ($P < 0.01$), and in the reduction of BA receptor expression ($P < 0.05$) (Fig. 2C).

As the persisting intestinal inflammation is usually associated to bacterial dysbiosis that is characterized by a substantial increase of bacterial groups with higher pro-inflammatory potential, such as the adherent-invasive *Escherichia coli* (AIEC) strains, we repeated the above experiment using the AIEC prototype, LF82, as inflammatory agent. Thus, Caco2 were challenged with LF82 (MOI10:1) for 3 hours. Again, this exposure resulted in the induction of inflammation in HepG2 cells, as shown by the increase of cytokines ($P < 0.01$), and in the reduction of BA receptors ($P < 0.05$) that returned to normal levels after LF82 removal (Fig. 2D).

Reducing Gut Inflammation Through the Anti-inflammatory Agent Dipotassium Glycyrrhizate Protects Liver Health by Improving Farnesoid-X-receptor and Pregnane-X-receptor Expression

DPG has been already studied and used in vitro and in vivo in our laboratory for its potent anti-inflammatory effects on cells from different tissues, including the gut. In this study, only Caco2 were exposed to cytomix for 48 hours or co-exposed to cytomix and DPG (150 and 300 μmol/L). We observed that DPG strongly reduced inflammation in HepG2, as shown by the decrease of IL-8 ($P < 0.01$), and significantly increased FXR and PXR expression dose proportionally ($P < 0.05$) (Fig. 3).

The mRNA Expression of Farnesoid-X-receptor, Pregnane-X-receptor, and TGR5 as well as Apical Sodium-dependent Bile Acid Transporter is Notably Altered in the Inflamed Colon of Children With Inflammatory Bowel Disease

There is a growing body of interest about a supposed role of BAs in mucosal immunity and inflammation. To assess a possible contribution of BA receptors in chronic inflammation, we analyzed the expression pattern of FXR, PXR, and TGR5 in inflamed colonic areas from children with CD, UC, and in age-matched controls. Moreover, we analyzed the expression of ASBT transporter that represents the first step in bile acid reabsorption to the liver from the intestine. We found a strong decrease of FXR, PXR ($P < 0.05$), and ABST ($P < 0.01$) in the inflamed mucosa of all patients as compared with controls. We also found an increased expression of TGR5 ($P < 0.05$) in the same patient samples as compared with controls (Fig. 4A and B), in agreement with results previously

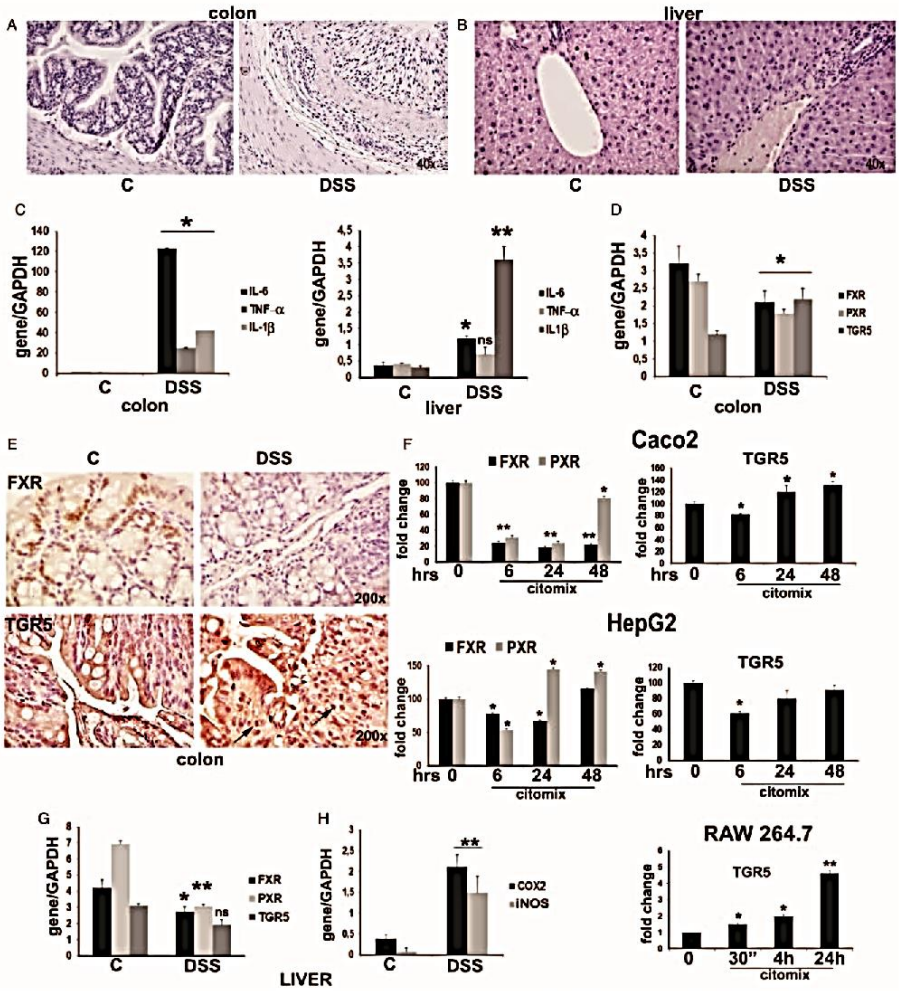

FIGURE 1. (A and B) Histological sections of colon and liver in DSS mice and control. (C) mRNA expression of IL-6, TNF-α and IL-1β in colon and liver and (D) mRNA level of FXR, PXR, and TGR5 in colon of DSS mice and control. (E) Immunohistochemistry showing FXR and TGR5 in colon of DSS mice and control. (F) mRNA expression of FXR, PXR, and TGR5 in Caco2 and HepG2 and TGR5 level in RAW 264.7. (G and H) mRNA analysis for FXR, PXR, and TGR5 and for iNOS and COX2 in liver of DSS mice and control. $^{*}P<0.05$; $^{**}P<0.01$. FXR = farnesoid-X-receptor; PXR = pregnane-X-receptor.

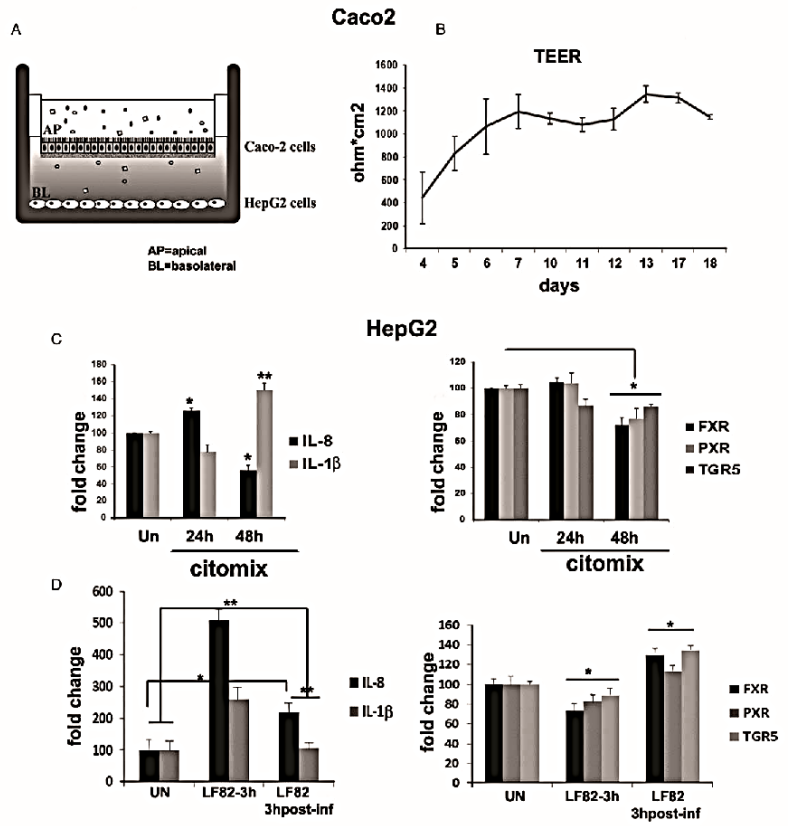

FIGURE 2. (A) Schematic representation of the co-culture model. (B) TEER measurement to control the integrity of epithelial barrier. (C) mRNA expression of IL-8 and IL-1β cytokines and FXR, PXR, and TGR5 receptors in HepG2 after 24 and 48 hours of treatment of Caco2 with cytomix. (D) mRNA expression of IL-8, IL-1β and FXR, PXR, TGR5 in HepG2 after 3 hours of LF82 infection and after 3 hours post-infection. $^{*}P < 0.05$; $^{**}P < 0.01$. FXR = farnesoid-X-receptor; PXR = pregnane-X-receptor; TEER = transepithelial electric resistance.

obtained in murine inflamed colonic tissues (Fig. 1D). Accordingly, immunohistochemistry showed that FXR was mainly concentrated in enterocytes and TGR5 in phagocytes (Fig. 4C).

DISCUSSION

Behind their role in nutrients absorption, Bas not only act as signaling molecules, activating several receptors that regulate central metabolic pathways but also may modulate inflammation. Accumulating data identify BAs and their receptors as pleiotropic-signaling molecules that control gut-liver crosstalk (36).

In this study, we first focused on the relationship between the gut and the liver, a close functional and vascular association also known as GLA, examining whether the occurrence of intestinal inflammation might affect healthy hepatic cells. Hence, we analyzed the liver of mice with a DSS-induced colitis and found that intestinal inflammation alters the BA receptor expression both in the inflamed colon and in the apparently normal hepatic tissue. Indeed, the cytokine analysis has proven the presence of a certain grade of inflammation also in the liver, as confirmed by histology. Interestingly, the nuclear receptors FXR and PXR were markedly down-regulated both in the gut and liver samples, whereas the transmembrane receptor TGR5 significantly increased in the gut but not in the liver of mice.

Recent data reported that TGR5 is highly expressed in monocytes/macrophages (35,37,38), members of the mononuclear phagocyte system that circulate through the blood and extravasate

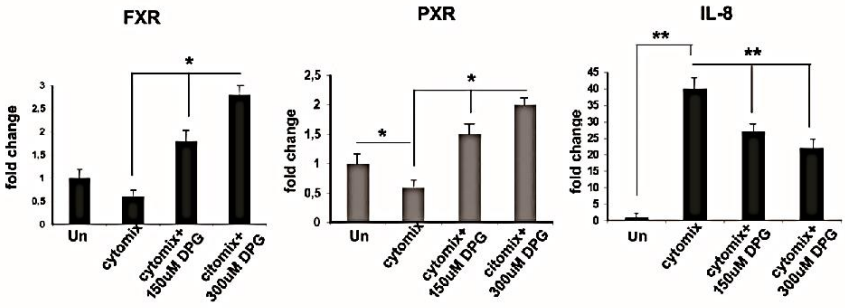

FIGURE 3. Farnesoid-X-receptor, pregnane-X-receptor, and IL-8 expression in HepG2 cells after a combined treatment of Caco2 with cytomix and 2 concentrations of dipotassium glycyrrhizate (DPG) (150 and 300 μmol/L) in a co-culture system. $^{*}P < 0.05$; $^{**}P < 0.01$.

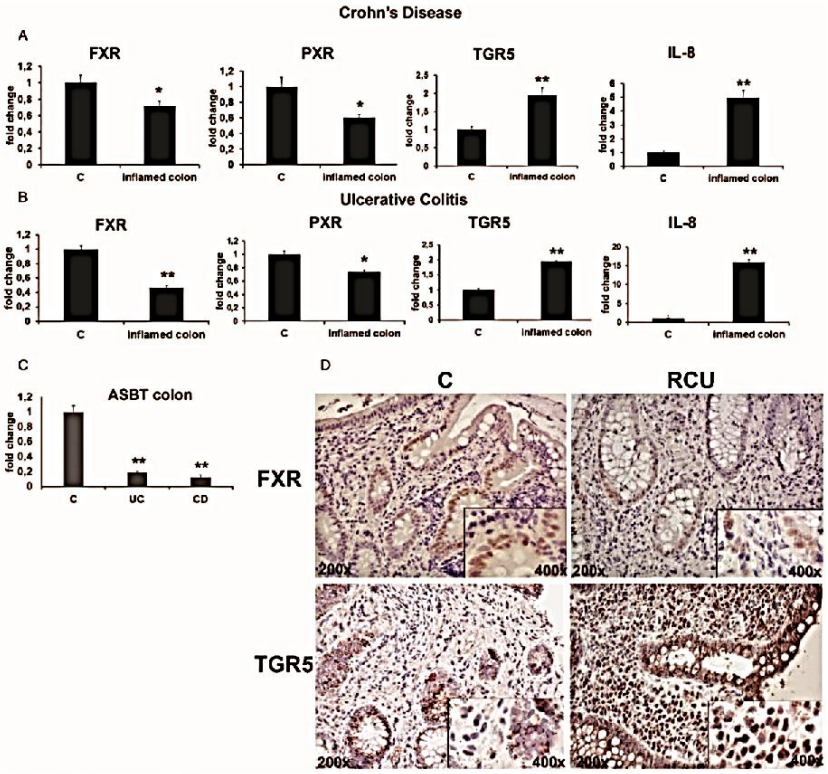

FIGURE 4. (A and B) Real-time PCR for the expression of FXR, PXR, TGR5, and IL-8 in inflamed colon of Crohn disease and ulcerative colitis pediatric patients. (C) mRNA level of ASBT in inflamed colon of Crohn disease and ulcerative colitis pediatric patients. (D) Immunohistochemistry for FXR and TGR5 in inflamed colon of a UC patient. CD, Crohn disease; UC, ulcerative colitis. $^{*}P < 0.05$; $^{**}P < 0.01$. FXR = farnesoid-X-receptor; PXR = pregnane-X-receptor.

into inflamed tissues. We performed immunohistochemistry and confirmed that TGR5 is principally localized in the macrophage infiltrate of murine colonic samples, whereas FXR is mainly found in the nuclei of enterocytes (in uninflamed tissue). To better investigate this different cell response, we induced in vitro inflammation in intestinal epithelial cells, hepatic cells, and macrophages and found that TGR5 was significantly increased only in the latter. This confirms that TGR5 is primarily expressed by macrophages and explains its marked increase in the inflamed gut, harboring the largest pool of macrophages.

As it is recognized that FXR has also antioxidant activities (39,40), we believe that the reduction of FXR in liver cells of DSS mice may also contribute to raise the oxidative stress level, as shown by the increased expression of COX2 and iNOS.

In summary, we found that gut inflammation induced by DSS alters BA receptor expression and causes liver inflammation in mice. It is, however, conceivable to attribute a share of damage to a direct action of the DSS that reaches the liver through the portal system. Therefore, in order to assess the extent of liver damage caused by intestinal inflammation as such, we used co-cultures of intestinal and hepatic cells. Intestinal cells, grown as a confluent monolayer mimicking the gut barrier were exposed to a mix of cytokines (TNF-α, INF-γ) as inflammatory agents. We found that the hepatic cells grown below showed increased levels of pro-inflammatory cytokines (IL-1β, IL-8) and a down-regulation of BA receptor expression, confirming that gut inflammation is able to elicit liver impairment.

Furthermore, there is a current widely agreed view that the gut microbiota (GM) plays a central role in BA host metabolism by regulating their deconjugation, dehydroxylation, dehydrogenation, and that BAs and GM reciprocally control each other's compositions (41,42). Additionally, it is known that intestinal inflammation is often associated with GM alterations leading to intestinal dysbiosis with a prevalence of adherent-invasive *E coli* (AIEC) pathotypes with pro-inflammatory properties (43). Hence, we used the AIEC prototype LF82 as an alternative agent to induce inflammation in intestinal cells and observed the same effects on liver cells as above. These results confirm that the onset and development of gut inflammation, featured by circulation of pro-inflammatory cytokines and abundance of AIEC strains, is also turned into a liver damage that should be seriously taken into consideration, given the chronic nature of some inflammatory processes, such as those characterizing the IBD.

Providing further evidence, we exposed intestinal cells to DPG, a salt of the glycoconjugated triterpene glycyrrhizin that exhibits potent anti-inflammatory, antiviral and antiallergic effects (44,45) and was previously used by our group to counteract gut inflammation (46). Remarkably, we observed that decreasing intestinal inflammation caused the reduction of liver inflammation and the normalization of BA receptor expression, strengthening the close gut-liver relationship.

BAs interact directly with a variety of transmembrane and nuclear receptors, in particular, FXR and TGR5 contribute to maintain the tolerogenic state of the liver and intestine immunity, indeed, they are highly expressed in innate immunity cells including intestinal and liver macrophages. Accordingly, perturbed BA circulation and/or metabolism seem to be implicated in the pathogenesis of primary sclerosing cholangitis, metabolic syndrome, colon cancer, and IBD (5,14). Thus, we analyzed the expression pattern of FXR, PXR, and TGR5 in inflamed colonic tissues of CD and UC children and found that FXR and PXR were significantly decreased, while TGR5 increased, in patients as compared with age-matched controls, in agreement with results in mice. Furthermore, as BAs secreted from the liver are mostly reabsorbed in intestinal epithelial cells via ASBT, we analyzed ASBT expression in the same samples and found that it was strongly decreased. We speculate that FXR, PXR, and ASBT decrease may result in intestinal BA accumulation worsening gut inflammation and causing liver distressing.

CONCLUSION

This study clearly demonstrates that gut inflammation, featured by the decrease of intestinal FXR, PXR, and ASBT expression and increase of TGR5, is able to affect hepatic cells by altering BA receptor levels and increasing the production of pro-inflammatory cytokines and oxidative stress. Hence, reducing gut inflammation is mandatory not only to improve the intestinal disease but also to protect the liver.

Future work will be addressed to understand whether gut inflammation is involved in the progression of liver diseases.

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2. INTRODUCTION

2.1 The gut-liver axis

The gut-liver axis (GLA) refers to the close bidirectional interface between the intestine and the liver, which occurs through the biliary tract, portal vein, and systemic circulation, arising from the integration of signals generated by dietary, genetic and environmental factors. This interdependent interaction is established through the vascular route of the portal vein that carries gut-derived products directly to the liver, and the liver feed-back route of bile and antibody secretion to the intestine.

The intestinal mucosal and vascular barrier is the functional and anatomical structure that serves as a playground for the interactions between the gut and the liver, limiting the systemic dissemination of microbes and toxins while allowing nutrients to access the circulation and to reach the liver **[1-4].**

The liver communicates to the intestine by releasing bile acids (BAs) into the biliary tract and systemic circulation **(Figure 1)**. In the gut, the host and gut microbiota (GM) metabolize endogenous (BAs) and exogenous (diet and environmental) substrates, whose products are brought to the liver through venous tributaries of the portal vein **[5, 6].**

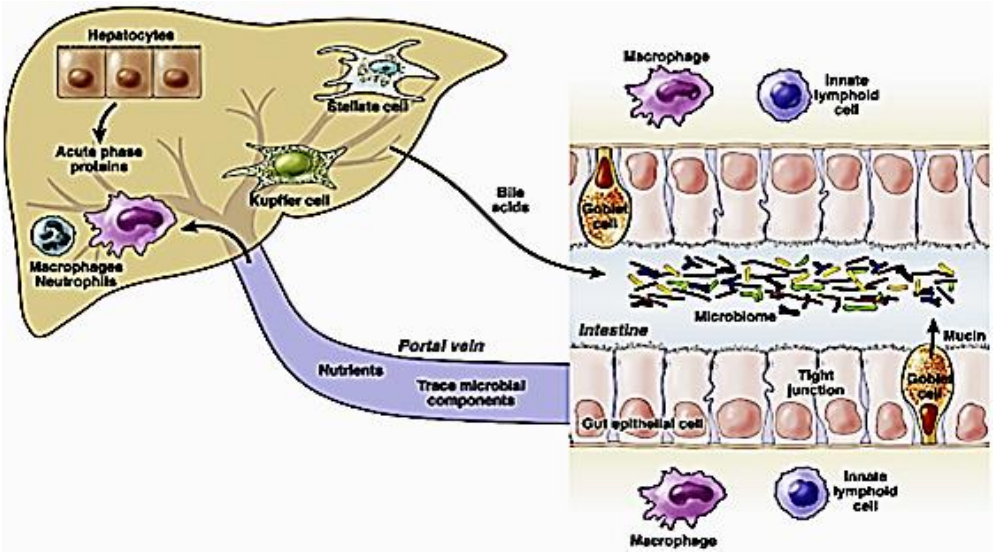

Figure 1: **The gut-liver axis**. The GLA connects the liver with the intestine via BAs metabolism. The anatomy of the liver provides its close interaction with the gut where nutrients and the microbiome contribute to the maintenance of a healthy metabolism and liver. From: Szabo, G., Gut–liver axis in alcoholic liver disease, Gastroenterology. (2015), **[7].**

The circulation of biliary acids, bilirubin, drugs or other substances from the liver to the bile, followed by entry into the small intestine, absorption by the enterocyte and transport back to the liver, is called "entero-hepatic circulation".

2.1.1 Entero-hepatic circulation of Bile Acids

BAs are amphipathic molecules synthesized from cholesterol. They act as detergents to expedite the digestion and absorption of dietary lipids and lipophilic vitamins in the gut **[8].**

Two pathways, the classic and alternative pathways, contribute to bile acid synthesis in humans **(Figure 2).** The classic pathway uses 17 cytochrome P450 enzymes (CYPs) to mediate de novo synthesis of primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), from cholesterol. The alternative pathway generates less than 10% of bile acids in the pool, and it produces CDCA by first oxidizing cholesterol to form 27-hydroxycholesterol, which is then hydroxylated by cytochrome P450-7B1 (CYP7B1), **[9, 10].**

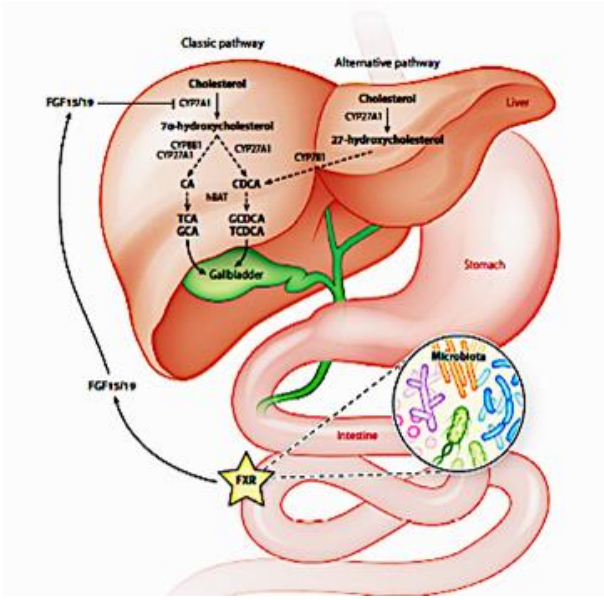

Figure 2: **Biosynthesis of bile acids**. BAs are synthesized in the liver from cholesterol via the classic or alternative pathway. The primary BAs are conjugated to either glycine or taurine prior to secretion into the gallbladder. Postprandial emptying of the gallbladder releases bile into the duodenum, where BAs are subject to interactions with the gut microbiota. From: Joyce, S. A., & Gahan, C. G., Bile acid modifications at the microbe-host interface: potential for nutraceutical and pharmaceutical interventions in host health, Annual review of food science and technology. (2016) **[11].**

Therefore pericentral hepatocytes produce primary BAs, which are successively conjugated to taurine or glycine and then released in the biliary tract. BAs, upon their arrival to the small intestine, facilitate the emulsification and absorption of fat-rich molecules and fat-soluble vitamins. Nearly 95% of BAs are actively reabsorbed in the distal ileum by the apical sodium-dependent bile acid transporter (ASBT) and transported back to the liver **[6, 12].** The residual 5% is converted to secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA), by the colonic microbiota (via deconjugation, dehydrogenation, and dehydroxylation) and passively reabsorbed into the portal circulation **[12].** Once in the liver, BAs are recycled and then secreted back to the biliary tract, completing the so-called enterohepatic circulation.

Aside from their important roles in digestion, BAs can behave as signaling molecules in carbohydrate and lipid metabolism, energy expenditure, and hepatic disease **[13-15].**
The regulatory functions of BAs are predominantly mediated by cellular receptors, such as the nuclear receptors (NRs) farnesoid X receptor (FXR) and pregnane X receptor (PXR) as well as the G protein-coupled receptor TGR5 **[16, 17].**

2.1.2 Bile Acid Receptors

Dedicated receptors for bile acids have been classified in two types: nuclear receptors and G protein-coupled receptors.
Nuclear receptors are ligand-dependent transcription factors that regulate a variety of physiological processes, such as physiological homeostasis, reproduction, development, inflammation and metabolism, by inducing the transcription of target genes. Upon activation by their ligands, NRs bind to their specific DNA elements, exerting their biological functions by regulating their target gene expression **[18].**

FXR is highly expressed in the liver and gut, relative to other tissues, and contributes to the maintenance of cholesterol/BAs homeostasis by regulating a variety of metabolic

enzymes and transporters. Intestinal intraluminal BAs regulate hepatic BAs synthesis by interaction with FXR, which induces transcription of an enterokine known as Fibroblast growth factor 19 (FGF19). This enterokine functions as a hormone, regulating bile acids synthesis, whit effects on glucose and lipid metabolism. In addition, FXR is a negative regulator of nuclear factor κB (NF-κB) -mediated hepatic inflammation and inhibited liver fibrosis, promoting regeneration [19, 20].

Similarly, the nuclear receptor PXR controls gene expression through the PXR responsive element module (PXRE) that is present in the promoter region of target genes [21]. PXR is highly expressed in the liver, small intestine and colon. This receptor has been traditionally involved in the regulation of xenobiotic metabolism, but recent evidences outlines its role also in the inflammatory response, cell proliferation and cell migration [22].

TGR5 receptor is a member of the rhodopsin-like superfamily member of GPCR protein and is ubiquitously expressed in diverse tissues, including endocrine organs, muscle, adipose tissue, immune cells, and intestinal tract [23]. TGR5 plays a role in regulating energy expenditure, glucose metabolism, and immunity. Activation of TGR5 in brown adipose tissue (BAT) and muscle positively regulates energy expenditure. TGR5 signaling is also involved in glucose homeostasis through its action in enteroendocrine L cells in the intestine. TGR5 activation in these cells induces release of glucagon-like peptide-1 (GLP-1), an incretin hormone that is secreted by L cells in both ileum and colon in response to luminal nutrients, such as carbohydrates and fats. In the pancreas, GLP-1 increases insulin synthesis and release. Given that TGR5 is expressed in mononuclear cells, including Kupffer cells, the resident macrophages of the liver, its activation in these cells appears to induce potent anti-inflammatory effects through inhibition of nuclear translocation of nuclear factor kappa B and suppression of cytokine production [24].

2.1.3 Intestinal Barrier

Generally, the gut constitutes a complex physical, chemical, functional and immunological barrier. Proceeding from the lumen inwards, its different components can be classified into the following levels: the microbiota, the extracellular elements, the epithelial cells, the immune system, the vascular structure (**Figure 3).**

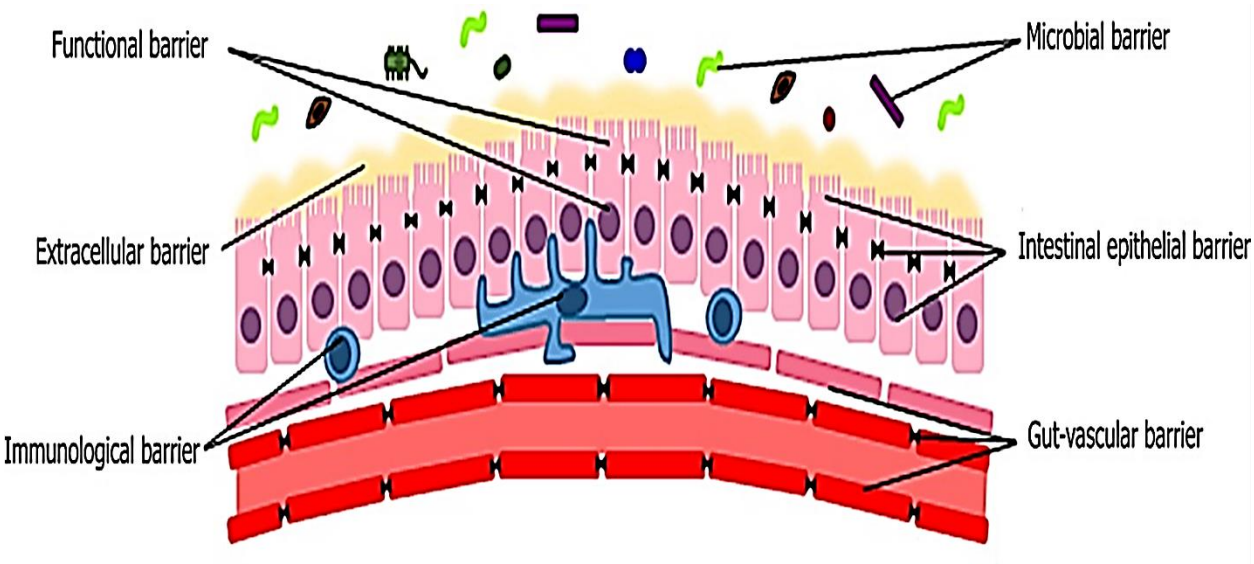

Figure 3: **Physiological gut barrier.** The intestinal barrier is a complex structure composed of the following four main barriers: microbiological, chemical, physical and immunological. These barriers play important roles in maintaining the stability of the internal and external environments. From: Nicoletti, A., et al., Intestinal permeability in the pathogenesis of liver damage: from non-alcoholic fatty liver disease to liver transplantation, World journal of gastroenterology. (2019) **[25].**

- *Microbial barrier*

The human GM harbors one hundred trillions of microorganisms.

Several factors, such as birth mode, age, diet and lifestyle, influence the human GM. In physiological conditions, its compositional and functional armony is quite stable over time. However, the onset of disease and/or the use of certain drugs can break this balance, resulting in dysbiosis. Indeed, the GM integrates the metabolism of the organism providing crucial pathways to process nutrients, vitamins and endogenous substances **[26]**. Microorganisms host in the lumen interact with the intestinal mucosa, shaping the mucus **[27]**, exerting a trophic and protective function towards enterocytes. Moreover, it

plays a pivotal role in the development, maturation and maintenance of the immune system [28-30] and induces local production of antimicrobial peptides (AMPs) and immunoglobulins [26-29].

- *Extracellular barrier*

Intestinal mucus is a gel formed by glycosylated proteins secreted by intestinal goblet cells called mucins. Mucus covers the whole gut and its thickness depends on the location, it prevents harmful substances and bacteria from directly contacting cell surface, causing inflammation [31, 32]. Thus, a regular structure of mucins is crucial for the maintenance of the gut barrier, and alterations could facilitate the absorption of harmful substances. Indeed, quantitative or qualitative alterations of the mucus layer has been documented in several diseases, such as inflammatory bowel disease (IBD) [32]. An increased mucus thickness has been related to alcohol intake and cirrhosis [33]. The inner side of the intestinal mucus is made of a fluid, which is not reached by the mixing forces of the luminal flow and peristalsis. The inner face of the mucin layer is devoid of bacteria [31] and directly contacts the intestinal epithelial cells, modulating the absorption of water and nutrients due to its static nature.

- *Functional barrier*

The external part of the mucus layer is continuously moved forward by peristalsis. The luminal flow prevents the proliferations of microorganism and a prompt clearance of detrimental elements. Gastric acid decreases microbial colonization of the small intestine. Only acid resistant microorganism, such as *Helicobacter pylori* and Lactobacilli are able to survive at low pH [34]. BAs have direct antimicrobial properties interfering with membrane and protein production and integrity [35, 36]. Thus, alterations of the bile and gastric fluid and impairment of the peristalsis cause modifications of the GM composition up to development of diseases [34, 37].

- *Intestinal epithelial barrier*

Under the intestinal mucus, there is a monocellular layer of enterocytes. Goblet cells, responsible for the production of the mucus, and Paneth cells, which produce AMPs, have additional functions and support to the homeostasis of the gut barrier. Enterocytes plasma membrane represents the main mechanical element of the mucosal barrier. In order to limit the gut permeability, intercellular spaces are sealed by the presence of a specific apical junctional complex, which is composed by a tight junction (TJ) and an adherens junction. Both tight and adherens junctions are connected to the cytoskeleton [38]. TJ are important elements for active and passive transport through the gut barrier. Both qualitative and quantitative alterations of TJ have been described in the context of liver disease [39, 40].

- *Immunological barrier*

The intestinal mucosal barrier is reinforced by the presence of a series of immune cells that contribute to the establishment of the barrier. In response to the exposure to bacteria and to their components, Paneth cells produce AMPs. Whenever microbial and pathogen-associated molecular patterns cross the intestinal barrier, they are identified through the interaction between pattern-recognition receptors, such as Toll-like receptors (TLRs) and nucleotide binding oligomerization domain-like receptors on the intestinal epithelial cells. Then, recruited dendritic cells are responsible for the transport of the captured antigens to the mesenteric lymph nodes (MLNs) for antigen presentation. This mechanism allows the priming and maturation of B and T lymphocytes, that become part of the adaptive immune response in the gut associated lymphoid tissue [41, 42].

- *Gut-vascular barrier*

Recent studies have revealed that the intestinal defense mechanisms actually go further, and also include a gut-vascular barrier (GVB) **[43]**. Spadoni *et al* **[43, 44]** hypothesized that a parallel structure in the gut could be responsible for the prevention of the translocation of bacteria and/or microbial components passed through the extracellular and the intestinal epithelial barrier. The basic structure of this entity is the gut-vascular unit. It is composed by the intestinal endothelium, which is associated with pericytes and enteric glial cells that surround it **(Figure 4).** The barrier is completed by TJ and adherens junctions, which are permeable to most of the small nutrients. Endothelial plasma membrane provides isolation and is equipped with active and passive transporters **[44, 45]**.

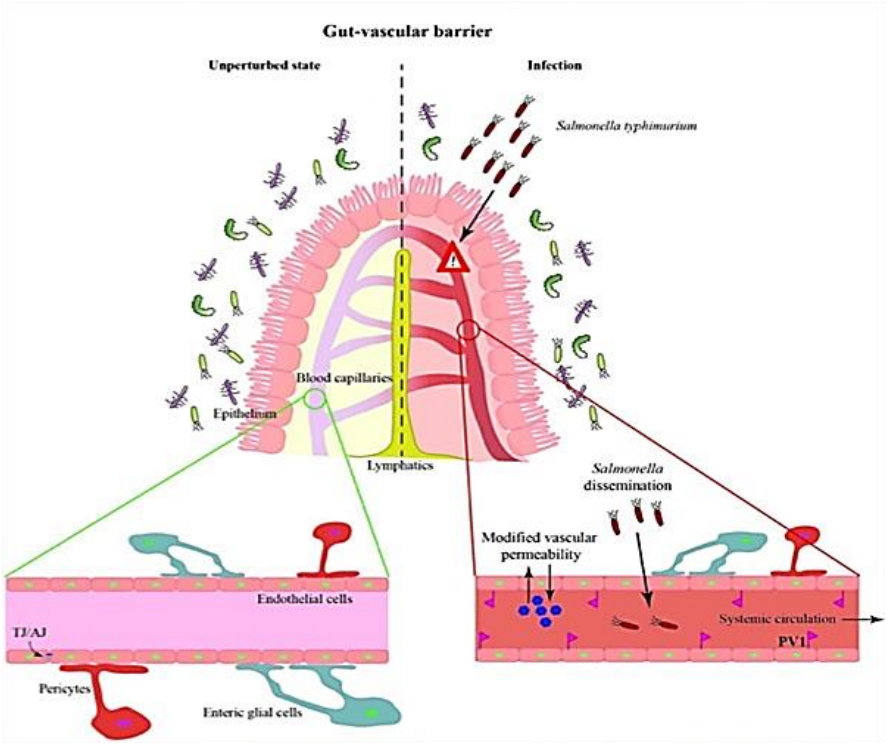

Figure 4: **Gut vascular barrier.** GVB controls the type of antigens that are translocated across the endothelial cells to reach the systemic circulation and prohibits entry of the intestinal microbiota. Intestinal pathogens (such as *Salmonella typhimurium*) can disrupt the GVB to favor their systemic dissemination. Indeed during infection gut endothelial cells show modified permeability to macromolecules and they up-regulate the expression of PV1 (Plasmalemma Vesicle Associated Protein-1), marker of leaky vascular barriers. From: Spadoni, I., et al., A gut-vascular barrier controls the systemic dissemination of bacteria, Science. (2015**) [43].**

Then the intestinal epithelial barrier is not a static physical barrier but rather strongly interacts with the cells of the immune system and the gut microbiome. Moreover, intestinal dysbiosis may favor intestinal barrier disruption and could be related to increased susceptibility to certain diseases **[46].**

2.2 Non-alcoholic fatty liver disease (NAFLD)

NAFLD is an umbrella term that comprises a continuum of liver conditions varying in severity of injury and resulting fibrosis.

Among these, hepatic steatosis (fatty liver) alone is referred to as NAFL (non-alcoholic fatty liver), and NASH (non-alcoholic steatohepatitis) is defined as a more serious process with inflammation and hepatocyte damage; typically, NASH is accompanied by percicellular fibrosis, which may progress to cirrhosis, this last damage is permanent and can lead to liver failure and liver cancer.

Epidemiology:

Globally, the prevalence of NAFLD is estimated at ~25% and is highest in the Middle East and South America and lowest in Africa **[47].** Whereas NAFLD typically is accompanied by central obesity in North America and Europe (~83% of patients), in Asia there is a sizable percentage of patients with 'lean NASH' who have a normal body mass index (BMI), even though the BMI cutoff for defining overweight in Asia (BMI > 23) is lower than in North America and Europe (BMI > 25) **[48].** Epidemiological analysis, based on other non-invasive techniques, indicate that about 30% of patients with NAFLD will progress to NASH. Among subjects with NASH approximately 20% will develop cirrhosis (normally patients with signs of ballooning degeneration and fibrosis) and of those with cirrhosis 30-40% will decompensate and die to liver-related complications over a 10-years period. A lower percentage (0-10%) progresses to liver cancer **[49, 50].**

Diagnosis:

Although NAFL or NASH can be strongly suspected in an individual on the basis of imaging and clinical features (such as the presence of metabolic comorbidities and abnormal lab tests), NASH can only be definitely diagnosed by liver biopsy; however, the risk associated with this procedure limits its use. For this reason, the prevalence of NASH is difficult to determine, as large population-based studies are not possible, indeed additional subgroups of NASH have been defined only recently **[51].**

Risk factors:

The incidence of NAFLD and liver disorders, such as fibrosis**,** increases with the age **[52].** The correlation seems to depend on to the higher prevalence of other risk factors **[53],** such as hypertension (HTN), obesity, and diabetes mellitus (T2D) that mediate the metabolic syndrome (MetS) set-up, associated with more severe biochemical, hematological and histological changes **[52].** Presence of MetS in an individual is the strongest risk factor for NAFLD and NASH **(Figure 6).**

MetS is variably defined, but typically includes increased waist circumference, hyperglycemia, insulin resistance (IR), dyslipidemia and systemic HTN**.** NAFLD is also associated with hormonal disorders (panhypopituitarism, hypothyroidism, hypogonadism, polycistic ovary syndrome), persistently elevated transaminases and hypoxia caused by obstructive sleep apnea, with some of these conditions predicting disease progression **[54-59].**

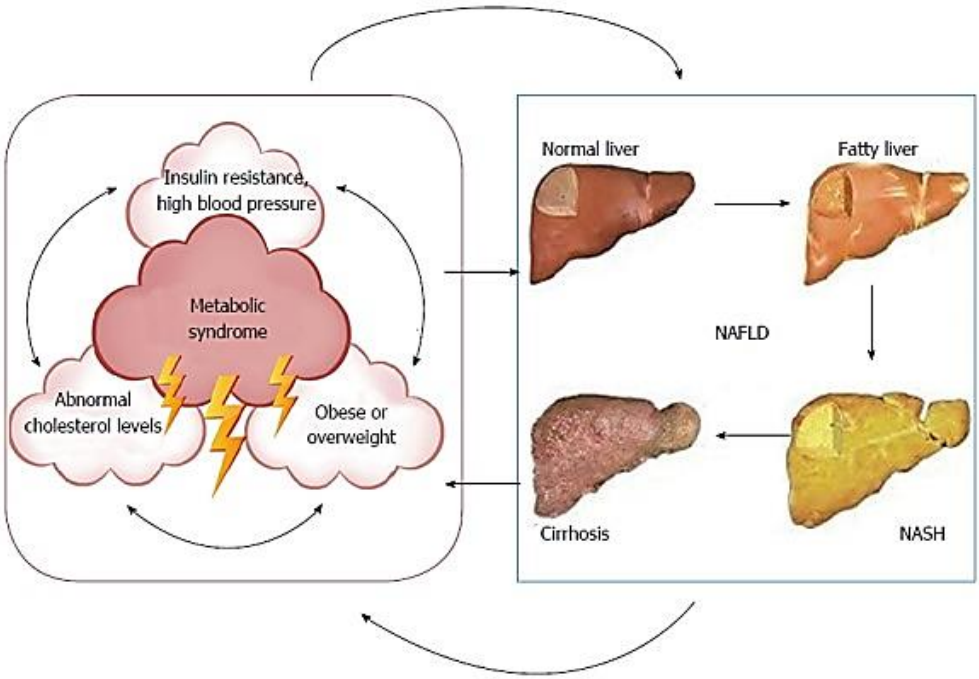

Figure 5: **The closed loop NAFLD-metabolic syndrome circuit.** This representation illustrates the mutual cause-and-effect relationship of NAFLD with the Metabolic syndrome. From: Lonardo, A., et al., Nonalcoholic fatty liver disease: Evolving paradigms. World journal of gastroenterology. (2017) **[60].**

Genetic risk factors of NAFLD are also known. Among all analyzed genes patatin-like phospholipase domain-containing protein 3 (PNPLA3, also called adiponutrin) and trans-membrane 6 super family 2 (TM6SF2) seem to play the most significant role **[61]**.

More recently, membrane bound O-acyltransferase domain-containing 7 gene (MBOAT7) has also gained attention.

More recently, a genome-wide association study revealed a splice variant (rs72613567:TA) in HSD17B13, a gene that encodes the hepatic lipid droplet protein 17β-hydroxysteroid dehydrogenase type 13, that was associated with reduced levels of alanine amino transferase (ALT) and aspartate amino transferase (AST), suggestive of less inflammation and liver injury in patients with fatty liver **[62].**

Pathogenesis:

A 'two-hit' theory was posited for several years to explain NASH pathogenesis **[63].** According to this theory in the first hit, a continuous and large nutrient intake can exceed the ability of the liver to metabolize the fats, determining a positive energy balance that

triggers the progressive intracellular accumulation of lipid droplets in the hepatocytes, leading to steatosis. The development of fatty liver is strictly associated with obesity and IR that predispose the liver to the progression to the second hit, which is characterized by necrosis, inflammation, and fibrosis. One of the mediators that determines the passage from the first to the second hit could be the oxidative stress **[63].** However, this view is now considered outdated.

There are many molecular pathways that contribute to the development of NASH, and it is not even certain whether NASH is always preceded by NAFL. Moreover, pathogenic drivers are not likely to be identical among all patients.

The steatohepatitis is the product of a complex interplay of several factors especially derived from adipose tissue and gut that would have an important role in the evolution of liver inflammation. Recent evidence highlights new concepts in clinical and pathogenic heterogeneity of NAFLD, a systemic disorder with a multifactorial pathogenesis and variable clinical manifestations. Other than the classical obese phenotype of NAFLD, a lean though metabolically abnormal variant has been recognized. Simple steatosis is no more considered a benign condition; IR is necessary but not sufficient for the disease progression, and NAFLD is not only a hepatic manifestation of metabolic syndrome, but may forerun the development of metabolic syndrome.

NAFLD and GM dysbiosis:

Lately there are also documented links between dysbiosis of the gut microbiota and liver diseaseas, in particular NAFLD.

The liver, as a 'first pass' organ exposed to the highest concentration of portal system products such as pathogen associated molecular patterns (PAMP), is the most vulnerable to their effects, particularly if pre- conditioned by a subclinical pathology such as lipid accumulation in hepatocytes.

Gut microbiota plays a role in the pathophysiology of metabolic diseases where NAFLD, through the gut-liver axis, is included.

A link has been established between intestinal microbiota abnormalities, barrier damage,

and hepatic inflammation and metabolic abnormalities under high-fat diet conditions. Increased intestinal permeability has largely been identified in mice on high-fat or choline-deficient diets and in patients with NAFLD **[39, 64-66].** The altered microbiota is directly responsible to disrupt the intestinal epithelial and vascular barriers as fecal microbial transplantation from high-fat diet fed mice to standard diet fed mice is sufficient to drive gut barrier damage, indicating that it is not the nutritional regimen, but its consequence on microbiota composition, that drives epithelial and GVB damage **(Figure 7), [66].**

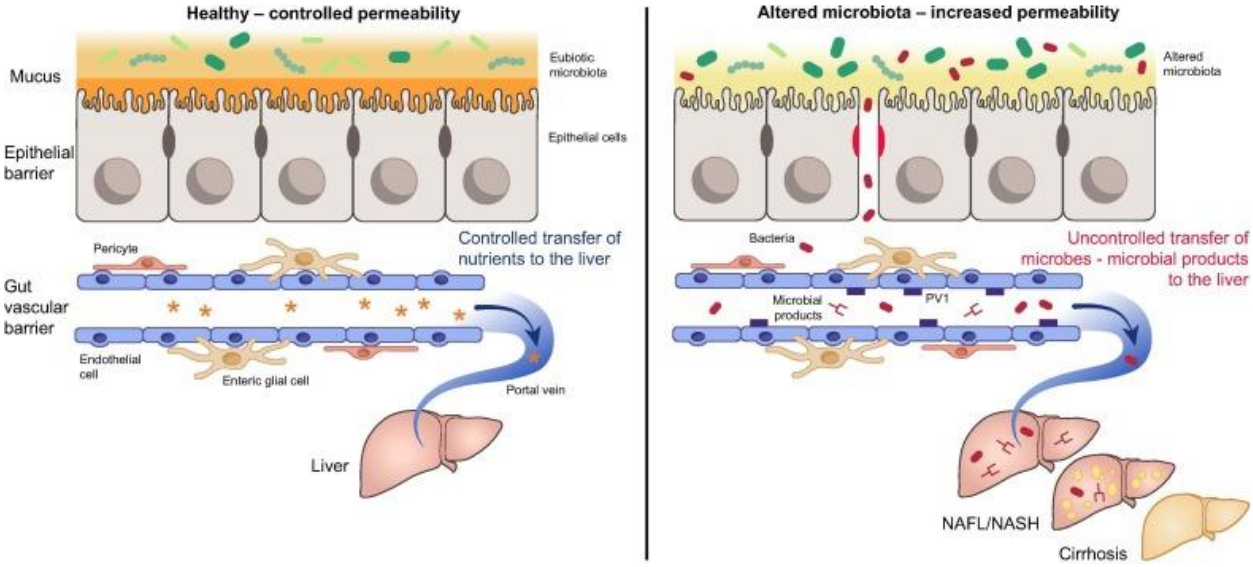

Figure 6: **Intestinal microbiota and Hepatic Health**. Left: Under homeostatic conditions the most external layer of defense is the mucus, just below, one can find the epithelium which is a monolayer of cells. A further layer is provided by GVB, which controls the systemic dissemination of microbial metabolites and the microbiota through the portal circulation. Right: Under inflammation, the intestinal barrier can be disrupted at several places, when the GVB is also damaged then the translocation of inflammatory microbial metabolites or microbes can occur to systemic sites, including the liver where they can induce local inflammation and promote liver disorders, such as NAFLD. From: Albillos, A., de Gottardi, A., & Rescigno, M., The gut-liver axis in liver disease: Pathophysiological basis for therapy. Journal of Hepatology. (2019) **[1].**

Similarly, patients with NAFLD show colon inflammation and reduced expression of the intestinal epithelial junction adhesion molecule Jam1 **[67].** Mice genetically deficient in Jam1 on a high-fat and fructose diet show increased intestinal permeability, endotoxinemia and hepatic inflammation, which emphasizes the importance of a healthy intestinal epithelial barrier to halt the portal entry of bacterial products under microbiota

dysruption.

The contribution of bacterial components to liver damage in NAFLD is supported by preclinical studies showing that hepatic steatosis, inflammation and fibrosis are attenuated in TLR-4 or TLR-9 deficient mice under a high-fat or choline-deficient diet [68, 69]. Further, inflammasome deficiency-associated changes in GM in mice results in hepatic steatosis and inflammation through portal influx of TLR4 and TLR9 agonists, leading to enhanced hepatic TNF-α expression and inflammation, which are especially severe in mice models of hepatic steatosis [70].

2.3 Gut barrier disruption: a prerequisite for NASH development

The pathogenesis of liver damage in patients with NAFLD is still incompletely understood, however a growing body of experimental and clinical data suggests a primary role of the gut-liver axis dysfunction **(Figure 8).**

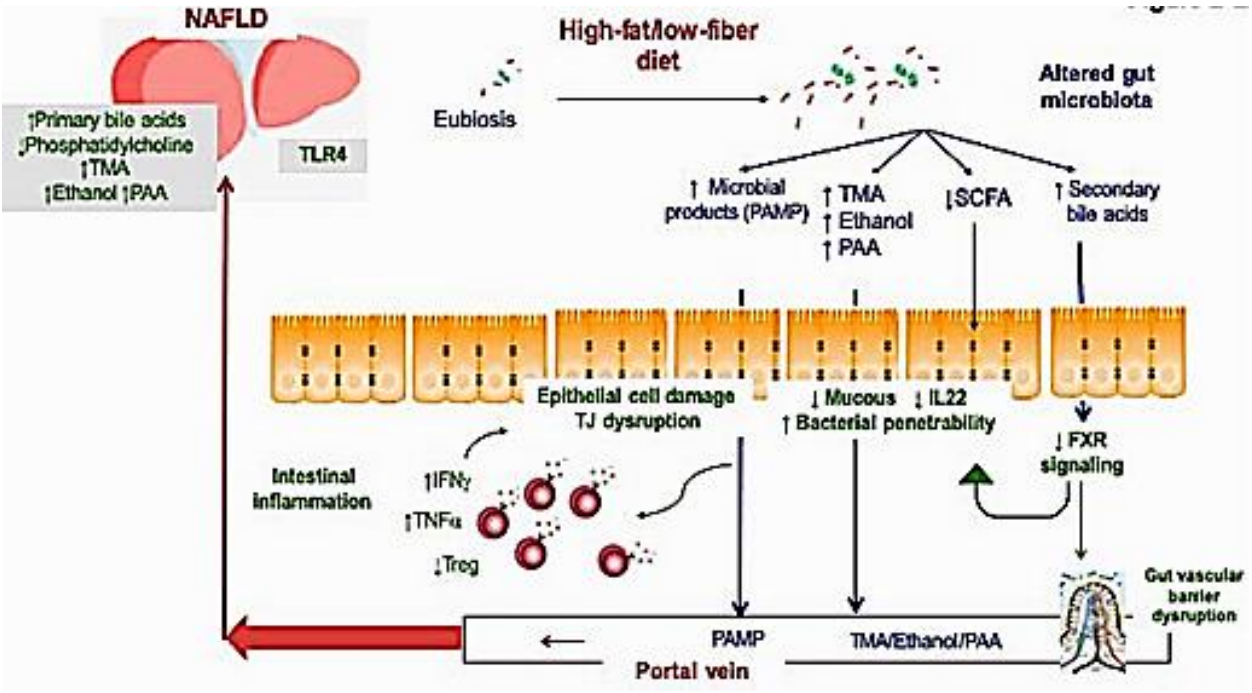

Figure 7: **Disruption of the gut-liver axis in NAFLD.** An altered GM is the cornerstone of gut-liver axis disruption in chronic liver diseases. Levels of secondary BAs are increased in the gut lumen as a consequence of alterations microbiota and an abundance of 7-alpha-dehydroxylating bacteria. This lead to reduced intestinal FXR-signaling. Consequences are loosening of epithelial cell intercellular junctions, mucous layer thinning and reduced synthesis of AMPs all of which facilitate bacterial penetrability and the interaction of pathobionts with mucosal immune system cells. From: Albillos, A., de Gottardi, A., & Rescigno, M., The gut-liver axis in liver disease: Pathophysiological basis for therapy. Journal of Hepatology. (2019) **[1].**

Brun *et al* **[71]** reported gut barrier dysfunction, tested as higher epithelial permeability to horseradish peroxidase in obese mice, both genetically deficient in leptin (C57BL/6Job/ob) and functionally deficient for the long-form leptin receptor (C57BL/6Jdb/db).

The intestinal mucosal chemical barrier refers to the gastric secretion of gastric acid, mucus, mucin, bile, glycoproteins, mucopolysaccharides, digestive enzymes, lysozymes, and other substances, which can alter the attack sites of pathogenic bacteria and act as a chemical barrier. The mucus layer contains AMPs, which help prevent contact between bacteria and the epithelium. The chemical barrier can protect the intestinal mucosa from erosion as a result of enzymes and acidic and alkali conditions. Gastric acid and bile can inactivate bacteria. The pH of gastrointestinal mucus and digestive juice is not conducive to the growth of bacteria. Gastric acid is the best bactericide in the gastrointestinal tract [72]. BAs can be combined with endotoxin; cholic acid can degrade endotoxin molecules; and lysozyme can destroy bacterial cell walls, destroying bacteria. The digestive juice secreted by the intestines can dilute the toxin and clean the intestinal cavity, making it difficult for the potentially pathogenic bacteria to adhere to the intestinal epithelium [73]. In addition, there are some complementary components in the intestinal secretion, which can help intestinal immune cells clear pathogens [38]. BAs can maintain the stability of the intestinal environment by inhibiting bacterial growth and translocation in the small intestine [74]. By activation FXR, bile acid negatively regulates the expression of sterol regulatory element binding protein 1c (SREBP-1c) [75] and reduces the expression of fat-related genes, reducing the occurrence of NAFLD [76].

The physical intestinal barrier is mainly composed of the intestinal mucous layer and epithelial cells. The junction complexes between epithelial cells include TJ, adherens junction, desmosome, and gap junction.

Studies have shown that increased intestinal mucosal inflammation and destruction of the intestinal epithelial barrier [77] lead to the possibility of translocation of microbial products, thereby inducing TJ proteins can produce contraction phenomena and move to the cytoplasm. The cell pore is clearly expanded, and the permeability of the intestinal mucosa increases, resulting in intestinal bacterial translocation and the release of metabolite lipopolysaccharides (LPS) into the blood and liver through the portal system [67], stimulating liver Kupffer cells and releasing inflammatory factors, such as TNF-α and IL-6 [78].

The inflammatory chemokines act on liver cells to make them liposomes.

A study found that the intestinal epithelium can also express innate immunity by regulating molecular TLRs [79]. Because immunoglobulin A (IgA) has a special affinity for Gram-negative bacteria in the intestinal tract, their function is obviously inhibited when the intestinal mucosa is damaged, thus promoting bacterial translocation in the intestine [80]. The LPS produced by bacterial metabolism is combined with the corresponding TLRs, producing PAMPs in the intestinal tract [81]. The activation of NF-κB and the mitogen-activated protein kinase (MAPK) signal transduction pathway stimulates the formation of inflammatory chemokines, such as TNF-α and IL-6, and leads to IR, which further promotes the development of NAFLD [82].

An abnormal intestinal barrier function can lead to disorder of digestion and absorption of nutrients, slow growth, reduced disease resistance, and increased susceptibility to pathogenic microorganisms and lead to the occurrence of various diseases [4].

When the intestinal epithelial TJ changes, decreases, or is absent, bacteria, toxins and macromolecules can enter in the systemic circulation. For example, some intestinal inflammatory diseases, such as inflammatory bowel syndrome (IBS) [83], are characherized by increased intestinal epithelial cell (iEC) bypass permeability.

In animal models of NAFLD, adaptation of a high-fat diet or high-fructose intake has been associated with increased gut permeability [84, 85]. Elevated concentrations of saturated fat or fructose favors pro-inflammatory microbiota; on one hand, suppressing production of SCFAs that are essential for intestinal barrier function, on the other hand recruiting macrophages and leading to the release of TNF-α and other cytokines causing mucosal inflammation [86, 87]. The consequence is a decreased expression of TJ proteins and a higher permeability of the gut barrier [88]. Diet-induced increases in blood LPS levels are known as metabolic endotoxaemia and play an important role in the activation of TLR-mediated low-grade liver inflammation, which are associated with NAFLD and NASH [89]. Current evidence from animal studies suggests that a high-fat diet or a high-fructose diet can induce metabolic endotoxaemia by altering the intestinal TJ proteins, mainly protein

zonulin-1 (ZO-1) and occluding **[71, 90-92].**

2.4 Current Therapies for NAFLD/NASH management

The currently disposable therapeutic alternatives for NAFLD/NASH can be divided into different categories: lifestyle modifications, pharmaceutical approaches, interventions on microbiome and interventions on intestinal content and mucus.
Furthermore, the study of food bioactive compounds is having great interest, due to discovery of some their hepatoprotective properties.

2.4.1 Diet and lifestyle interventions

It is well accepted that obesity is one of the principal risk factors for the development of fatty liver. For this reason, weight reduction, dietary changes, and physical exercises are considered the gold standard for the reversion of NAFLD/NASH. It is generally known that the level of energy intake is higher in patients with NAFLD than in healthy subjects and the calories are mainly consumed in form of carbohydrates, saturated fats and cholesterol. An excess of carbohydrates affects glucose and free fatty acids metabolism in the liver, on the contrary, a restriction in the consumption determines weight loss and improvement of lipid profile **[93].** Histologically, steatosis, inflammation, and fibrosis get better, confirming the efficacy of carbohydrate limitation in the reversion of NASH **[94].**
Dietary lipid content has also been identified as an important factor in the development of NASH. Specific type of fats has a greater impact on the progression of simple steatosis or in its improvement. Polyunsaturated fatty acids (PUFA), mainly n-3 and mono-PUFA, exert a protective role in fatty liver, determining adiponectin level increase and a reduction of serum insulin, triglycerides and leptin amount **[95].**

2.4.2 Pharmaceutical interventions

Usually, drug interventions are based on the association of several compounds in order to reverse the co-morbidities that characterized the metabolic syndrome.

Antioxidant. The amount of reactive oxygen and nitrogen species, generated by lipoperoxidation, could exceed the capacity of the cellular antioxidant systems, leading to oxidative stress. It has been supposed that this condition is responsible for the progression from NAFLD to NASH. Several antioxidants, as Ursodeoxycholic acid (UDCA) **[96],** are normally introduced in NAFLD management to counteract the production of these reactive compounds, in order to prevent the damage progression.

Insulin sensitizers. This class of drugs, including metformin and thiazolidinediones (TZDs- such as pioglitazone and rosiglitazone), improves hepatic insulin activity. The improvement in insulin sensitivity by metformin could be ascribed to its positive effects on insulin receptor expression and tyrosine kinase activity **[97].** It has also been reported that metformin acutely increases plasma levels of GLP-1 and induces islet incretin receptor gene expression through a mechanism that is dependent on peroxisome proliferator-activated receptor (PPAR)-α **[98].** TZDs reduce IR in adipose tissue, muscle, and liver.

Lipid lowering drugs. The main drugs used to reduce the plasma levels of cholesterol are statins **[99].** The beneficial effects of these molecules depend on their capacity to reduce cholesterol biosynthesis, mainly in the liver, derived from an inhibitory effect on HMG-CoA reductase.

Pharmaceutical compounds in development. Obeticholic acid (OCA), a ligand of FXR, is a synthetic variant of natural bile acid chenodeoxycholic acid. In animal models, FXR activation has been demonstrated to reduce hepatic glucogenesis, lipogenesis, and steatosis. In the FLINT trial, treatment with OCA achieved a primary end-point of improving the necro-inflammation without worsening of fibrosis in 46% of the treated patients with NASH. Moreover, compared to placebo, NASH resolution was obtained in 22% of treated patients **[100].**

2.4.3 Interventions on microbiome

There is growing evidence that the GM is the real goal of NAFLD interventions. In view of the key role of the GM in the pathogenesis of metabolic diseases, [101, 102] the design of gut microbiota regulation strategies to improve NAFLD may be considered as a new treatment option for these patients. In this respect, probiotics, prebiotics, synbiotics and fecal microbiota transplantation have attracted attention.

- *Probiotics* is a group of complex bacteria [103]. Commonly used probiotics include Lactobacillus, Bifidobacterium and Polycoccus, which can inhibit the expansion of Gram- negative pathogens and have a wide range of beneficial effects on host metabolism [104, 105]. Some evidence has indicated that probiotics have the ability to improve liver damage and reduce bacterial translocation [106] by improvement of the integrity of the epithelial barrier and stimulate the host immune response [107, 108]. Furthermore, probiotics have synergistic effects with chemical drugs, such as statins, in the treatment of NAFLD, which highlights the great potential of probiotics alone or in combination with other drugs [109 ,110].

- *Prebiotics,* which contain no living microorganisms, are non-digestible food ingredients that can selectively promote the proliferation and/or activity of one or several gut microbes [111]. *Synbiotics* are combination of prebiotics and probiotics [112]. At present, probiotics and prebiotics play a key role in the treatment and prevention of NAFLD.

- *Fecal microbiota transplantation (FMT)* is a new approach to clinical treatment, in which gut microbes are transferred from healthy donor to diseased recipient. By this way, a 'healthy' gastrointestinal microbiota may be reconstructed. Zhou and co-workers found that FMT intervention remarkably increased the concentration of butyrate in fecal contents and improved the TJ of small intestine. This study further proved that FMT attenuated steatohepatitis in mice by a beneficial regulation of GM [113].

2.4.4 Interventions on intestinal content and mucus

A completely different approach for NASH management involves using poorly absorbable, adsorptive material to bind gut-derived toxins and bacterial products, thus abrogating their inflow into the liver and systemic circulation.
Recent advances in activated carbon technology have led to the development of synthetic adsorptive nanoporous carbons. They have uniquely tailored porosity that is acquired during synthesis and activation **[114].**

2.5 Glycyrrhizin and its effects on GLA

Plant drugs are known to play a key role in the management of liver diseases. There are many plants and herbal extracts that have demonstrated to possess hepatoprotective activities. However, only a small proportion of hepatoprotective plants, as well as formulations used in traditional medicine, is pharmacologically evaluated for their safety and efficacy **[115 ,116].**

Glycyrrhizin (or glycyrrhizic acid or glycyrrhizinic acid) is the chief sweet-tasting constituent of Glycyrrhiza glabra (liquorice) root and has been used in traditional medicine to alleviate bronchitis, gastritis and jaundice. Its aglycone is enoxolone appreciated as a prodrug used in Japan to reduce the risk of liver cancer in people with chronic hepatitis C **[117, 118].**

Structurally, glycyrrhizin is a triterpenoid compound used as an emulsifier and gel-forming agent in foodstuffs and cosmetics. Glycyrrhizic acid is composed of a hydrophilic part, two molecules of glucuronic acid, and a hydrophobic fragment, glycyrrhetic acid **[119]**, **(Figure 9).** The yellow color of licorice is due to the flavonoid content of the plant, which includes liquiritin, isoliquiritin, isoflavones, glabridin and hispaglabridins. The

hispaglabridins A and B have significant antioxidant activity **[120],** and glabridin and glabrene possess estrogen-like activity **[121].**

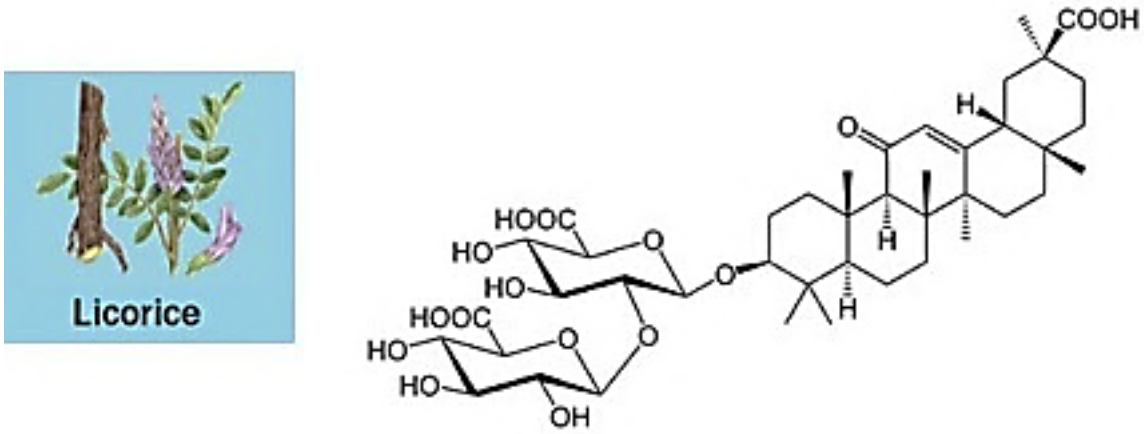

Figure 8: **Licorice root and chemical structure of glycyrrhizin.** From: Omar, H. R., et al., Licorice abuse: time to send a warning message. Therapeutic advances in endocrinology and metabolism. (2012) **[122].**

Glycyrrhizin has a poor oral bioavailability and is detected at very low levels after a single oral dose administration. After oral ingestion of licorice in humans, glycyrrhizic acid is hydrolyzed to glycyrrhetic acid by intestinal bacteria possessing a specialized ß-glucuronidase **[123].** Glycyrrhetic acid is a 200–1000 times more potent inhibitor of 11-ß-hydroxysteroid dehydrogenase (11-ß-HSD) than glycyrrhizic acid; therefore, its pharmacokinetics is more relevant after oral administration. Glycyrrhetic acid is then rapidly absorbed and transported via carrier molecules to the liver. In the liver it is metabolized to glucuronide and sulfate conjugates which are transported efficiently and excreted into the bile and are then subjected to entero-hepatic circulation **[124].** These conjugates are subsequently hydrolyzed by commensal bacteria **[124, 125].** The transit rate of gastrointestinal contents through the small and large intestines predominantly determines to what extent glycyrrhetic acid conjugates will be reabsorbed.

Dipotassium glycyrrhizate (DPG) and diammonium glycyrrhizinate (DG) are salts of glycyrrhizin which are usually used for oral formulations because of their greater solubility.

It has been reported that glycyrrhizin possesses anti-inflammatory and antioxidant activities and can stimulate endogenous production of interferons [126]. Antifibrotic activity of glycyrrhizin could be attributed to its inhibitory activity on NF-κB [127]. Liang et al. showed that glycyrrhizic acid induces inhibitory effects on hepatocyte apoptosis and liver fibrosis, which were associated with downregulation of connective tissue growth factor, hepatic stellate cells activation. These effects may provide potential therapeutic strategies for fibrosis [128].

Lately, the beneficial activities of glycyrrhizin in the intestinal district have attracted a lot of interest. A recent study shows that DPG exerts inhibitory effects against high-mobility group protein-1 (HMGB1) activity, significantly reducing intestinal inflammation [129].

Thus, DPG could represent an innovative tool for controlling intestinal inflammation and improving mucosal healing.

In addition other scientists have observed the ability of DG to improve the expression of TJ proteins, the goblet cell number, and mucin secretion and sequentially to enhance the function of intestinal barrier in a NAFLD mouse model. It can significantly alleviate the intestinal mucosa inflammation in mice [130].

3. AIMS OF THE SECOND PHASE OF EXPERIMENTATION

Recent findings suggest that the occurrence of gut inflammation may affect the severity and progression of liver disease, in particular of NAFLD, one of the most important causes of liver disease worldwide, and its progressive form NASH, that in turn may develop into cirrhosis and hepatocarcinoma. The lack of preclinical models of progressive NAFLD/NASH in presence of gut inflammation, that recapitulates the human disease, is a barrier to the pathogenesis comprehension and innovative therapeutic strategy development. Furthermore, despite several agents are under development, however, there are no drugs currently approved for NAFLD/NASH treatment. Glycyrrhizin is the major component of licorice root with multiple biological activities including antioxidative, antiinflammation, anticancer and antiviral. DPG, a salt of glycyrrhizin, should represent a novel strategy to improve NAFLD/NASH directly or by reducing gut inflammation.

In this frame, this study aimed to:

1. develop an animal model of hepatic steatosis, displaying the disease both in its early (NAFLD) and late (NASH) phase, in which an important intestinal inflammation was also induced;
2. use this model to assess that gut inflammation significantly contributes to the severity and progression of the liver disease (from NAFLD to NASH) by increasing inflammatory (IL-6, TNFα, NLRP3, TLR4, MCP-1, HMGB1) as well as fibrotic (TGF-β, α-SMA) mediator expression and altering BA receptor expression;
3. evaluate the potential of the anti-inflammatory molecule, the DPG, to improve the liver disease by reducing gut inflammation.

4. MATERIALS AND METHODS

4.1 Animals

C57BL/6J male mice (6 weeks of age) were purchased from the animal housing unit of Envigo RMS, Srl. Mice were housed in collective cages at 22° ± 1° C under a 12-hour light/dark cycle and with food and water provided *ad libitum*.

4.2 Experimental design and animals treatment

A total of 50 mice were fed with a normal diet for 1 week.

After the adaptation period, animals were randomly divided into 4 groups: (1) control group was given a standard regular diet and tap water *(n=8);* (2) NAFLD/NASH group was given a high-fat diet (HFD) plus high fructose/glucose in drinking water *(n=14);* (3) NAFLD/NASH-DSS group was given a HFD plus high fructose/glucose and cyclic administration of dextran sodium sulphate (DSS) 1% (w/v) in drinking water *(n=14);* (4) NAFLD/NASH-DSS-DPG group was given an HFD plus high fructose/glucose, DSS 1% and the mice were treated with DPG *(n=14).* Mice were sacrificed at two times: 8 and 13 weeks.

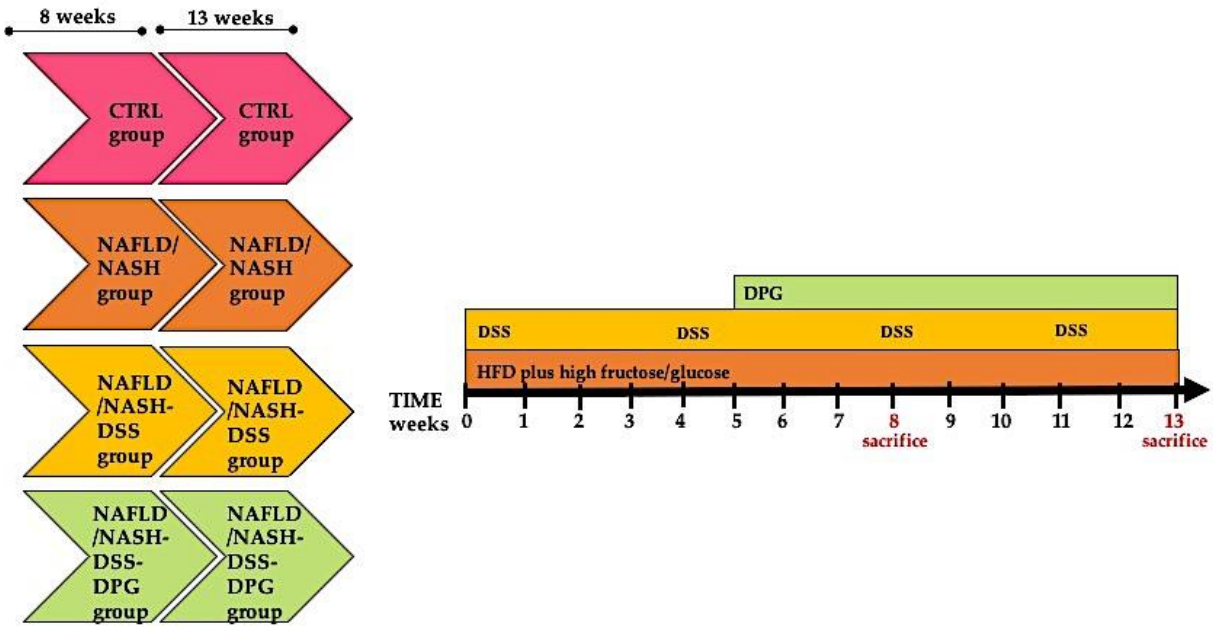

Figure 9: Graphical representation of experimental design.

Induction of NASH. The HFD was provided from Laboratorio Dottori Piccioni, Milan, Italy. The HFD consisted of 18% protein kcal, 24% carbohydrate kcal, 58% fat kca for a total of 5.6 kcal/g of diet. Our protocol also provided the addition of 42 g/L of carbohydrates mixed with water in a ratio of 55% fructose and 45% glucose (23.1 g L-fructose plus 18.9 g L-glucose in 1 l of tap water; Sigma-Aldrich, St Louis, MO, USA).

Induction of intestinal inflammation. The mice were treated with four cycles (each of 7 days) of 1% dextran sodium sulfate (DSS, molecular mass, 36,000–50,000 Da, MP Biomedicals, Santa Ana, CA), dissolved in autoclaved drinking water.

Intervention on intestinal and hepatic inflammation. Since the fifth week the selected dose of 8 mg/kg/day DPG (DMG Italia Srl, Pomezia, italy), diluted in Phosphate Buffered Saline (PBS), has been administered by oral gavage.

Body weight, stool consistency and presence of fecal blood were recorded weekly. After sacrifice, the colon was removed and examined for weight and lenght, the liver assessed only for weight. Livers and colons were collected for histological and transcriptomic study, serum and fecal samples were collected and stored at -80°C for biochemical and molecular analyses.

4.3 Ethic Statement

The experimental procedures were previously approved by the Ministry of Health for the protection of animals used for experimental purposes, and the study was conducted in accordance with Italian regulations on animal welfare. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Italian National Agency for New Technology, Energy and Sustainable Economic Development (ENEA). The general health of the animals was monitored daily by the Veterinarian in the animal facility.

4.4 Disease Activity Index (DAI)

The DAI scores were calculated according to loss of body weight, stool consistency and gross bleeding, and exhibited as the mean value of the following three parameters: (a) body weight loss: 0 = none; 1 = 1–5%; 2 = 5–10%; 3 = 10–15%; 4 = over 15%; (b) stool consistency: 0 = normal; 2 = loose stools; 4 = diarrhea; (c) gross bleeding: 0 = normal; 2 = hemoccult; 4 = gross bleeding.

4.5 Histological analysis and NASH scoring

Colonic and hepatic samples were fixed in 10% formalin and embedded in paraffin for routine histology. Four μm sections were mounted on slides and stained with standard hematoxylin and eosin (H&E) techniques.

Colonic sections were analyzed by light microscopy and scored according to the criteria of Maxwell *et al.* **[181]**.

Hepatic sections were examined to semi-quantitatively assess the NAFLD activity score (NAS) based on histological features. The score is in three categories and classified into: steatosis (0–3), lobular inflammation (0–3), and hepatocelular ballooning (0–2). If NAS of a liver specimen is ≥5, the section is defined as "NASH". If NAS is smaller than 3, it represents as "non-NASH" **[182].** The extent of liver fibrosis was assessed after Masson's Trichrome staining (cat. no. HT-15; Sigma-Aldrich), and semi-quantitatively scored between 0 and 4 following the criteria as reported previously **[182, 183].** Experiments were carried out in blind.

4.6 Stainings for the total mucins and assessment of the mucus thickness

Periodic Acid – Schiff's (PAS) and Alcian blue (AB) stainings were used to visualize the total (neutral and acid) mucins in colon. Dewaxed colon sections were stained using a NovaUltra™ Alcian Blue Stain Kit (cat. number – IW3019 - IHC World, Woodstock, MD) following the supplier's instructions and they were analyzed by light microscopy.

4.7 Immunofluorescence

Paraffin-embedded intestinal sections of 4 μm were rehydrated, blocked with 5% BSA in PBS with 0,3% Triton X-100 and stained with the anti-ZO1 (1:100, Invitrogen,33-9111) and anti-PV1 (1:100, BD Pharmingen™, 553849)) antibodies following the manufacturer's protocol. Before imaging, nuclei were counterstained with DAPI.

4.8 Immunohistochemistry

Immunohistochemical staining (IHC) on 4 μm thick paraffin-embedded liver sections was performed following the standard protocol.

Briefly, sections were dewaxed for 20 min at 56 °C and incubated in citrate buffer pH 6.0 for 20 min at 95°C. Afterward, sections were washed in water for 5 min and peroxidases inhibited by incubation in 3% H2O2 for 10 min. Sections were treated with 5% bovine serum albumin (Santa Cruz) for 20 min and incubated with primary anti-F4/80 (1:150, Abcam, Cambridge, UK) and anti-HMGB1 (1:1000, Sigma-Aldrich, St. Louis, MO) antibodies in phosphate-buffered saline for 1 h at room temperature in a moist chamber. They were then washed in phosphate-buffered saline, incubated for 30 min with the secondary anti-rabbit antibody (Dako North America, Carpinteria, CA), and washed again in phosphate-buffered saline. The DAB detection kit (DAKO) was used, as suggested by the providers, to visualize the antigen. Finally, samples were stained with H&E.

4.9 Liver tissue lipid content

Liver biopsies were embedded in OCT compound gel. Frozen liver sections of 8-μm thickness were fixed in 4% neutralized paraformaldehyde in PBS and stained with Oil Red-O (ORO) (cat. no. O0625, Sigma- Aldrich) to visualize fat droplet deposition in hepatocytes.

4.10 Analysis of serum parameters

As a measurement of liver damage, murine serum alanine aminotransferase (ALT) activity was measured using a Alanine Aminotransferase Activity Assay kit (MAK052, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

4.11 Real-Time Polymerase Chain Reaction

Total RNA was isolated from mouse colonic/hepatic tissues using the mini RNeasy kit (Qiagen) and 1μg of total RNA was reverse transcribed by IscriptTM cDNA Synthesis Kit (Biorad, Hercules). The RT-PCR amplifications were obtained by a BioRad CFX96 TouchTM Real-Time PCR Detection System using SsoAdvanced Universal SYBR Green super Mix (Biorad). The primers used were summerize in **Table 1**. Messenger RNA (mRNA) levels were normalized to those of GAPDH for mice samples by the $2^{-\Delta\Delta CT}$ method. The expression level of each mRNA was reported as folds of induction as respect to controls.

Symbol	Gene name	Forward	Reverse
IL-6	*Interleukin-6*	CAAGTCGGAGGCTTAATTACACATG	AGAAAAGAGTTGTGCAATGGCA
TNF-α	*Tumor necrosis factor-α*	CAGACCCTCACACTCAGATCATCTT	TCGTAGCAAACCACCAAGTGG
NLRP3	*NOD-, LRR- and pyrin domain containing protein 3*	TGCTCTTCACTGCTATCAAGCCCT	ACAAGCCTTTGCTCCAGACCCTAT
TLR4	*Toll-like receptor 4*	CGCTTTCACCTCTGCCTTCACTACAG	ACACTACCACAATAACCTTCCGGCTC
α-SMA	*α-Smooth Muscle Actin*	ACCATTGGAAACGAACGCT	TTTCGTGGATGCCCGCTG
TGF-β	*Transforming Growth Factor -β3*	GTGGAAATCAACGGGATCAG	ACTTCCAACCCAGGTCCTTC
PXR	*Pregnane XReceptor*	GATGGAGGTCTTCAAATCTGCC	CAGCCGGACATTGCGTTTC
RPL32	*Ribosomal protein L32*	TGTGCAACAAATCTTACTGTGCT	TGCACACAAGCCATCTACTCA
MCP-1	*Monocyte chemoattractant protein-1*	AACTGCATCTGCCCTAAGGT	CTGTCACACTGGTCACTCCT

Table 1

4.12 Fluorescence *in situ* hybridization

For fluorescence *in situ* hybridization (FISH), paraffin-embedded liver sections were incubated with 10 mg/ml lysozyme (Sigma) in Tris-HCl 0.1 M (pH 7.4) for 30 min. Slices were then washed and incubated with 5ng.ml $^{-1}$ of probes (mixed Eub338 and non-Eub in hybridization buffer (0.9 M NaCl, 0.1% SDS) at 50 C for O/N in a humid chamber. A mix of probes were used, as shown in **Table 2.**

Samples were washed with Tris-HCl 0.1 M (pH 7.4) and blocked with Tris-HCl 0.1 M pH 7.4 (2% FBS, 0.3% Triton X) for 30 min at room temperature. Then, they washed and, before imaging, were counterstained with DAPI. For each mouse, 4 to 8 images were taken, and the total number of bacteria was determined.

GENE	FLUOROCHROME	SEQUENCE
Eub338 I	FICT	5'- GCT GCC TCC CGT AGG AGT - 3'
Eub338 II	FICT	5'- GCA GCC ACC CGT AGG TGT - 3'
Non-Eub	Alexa 647	5'- ACT CCT ACG GGA GGC AGC - 3'

Table 2

4.13 Fecal Extraction

Murine stool specimens, stored at -80°C, were resuspended in extraction buffer (ScheBo Biotech AG, Giessen, Germany) to a final concentration of 500 mg/mL. Samples were vortexed for 1 minute at room temperature and placed in orbital shaking for 1 hour at room temperature. After being centrifuged twice for 5 minutes at 10,000 rpm at 4°C, clear supernatants were collected and stored at -80°C. Total protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

4.14 Immunoblot Analysis

Fecal extract (10μg) was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to detect selected proteins. Proteins were transferred in polyvinylidene fluoride membrane (Bio-Rad) and blocked with TBS-T (Tris- buffered saline with Tween-20) containing 5% non-fat dry milk. Anti-HMGB1 (1:1000; R&D system; Sigma) antibody was diluted in TBS-T containing 3% non-fat dry milk and incubated overnight at 4◦C. Membranes were washed in TBS-T, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.), washed in TBS-T, and developed with ECL-Plus (GE Healthcare, Life Science). Densitometrical analyses of the blots were performed using the Software ImageQuant (GE Healthcare, Life Science).

4.15 Statistics

From 4 to 7 mice per group were included in all experiments to ensure statistical significance, as the model is reproducible, and the mouse population is homogenous. Statistical analysis for significance was determined using the GraphPad InStat software. The values in different experimental groups were compared using either Student's t test or one-way ANOVA and are expressed as means ± standard deviation. A value of $P<0.05$ was considered to be statistically significant between different groups. Experiments were repeated 3 times. Differences were noted as significant *$P < 0.05$, and **$P < 0.01$.

5. RESULTS

5.1 Set up and characterization of a murine model of NAFLD/NASH with intestinal inflammation

We set up a NAFLD mouse model with a progressive disease and with intestinal inflammation by feeding C57BL/6J mice with high fat and high sugar diet for different times to develop liver steatosis and steatohepatitis with fibrosis and, in addition, by administering mice with cyclic treatments of DSS 1% to develop gut inflammation. Mice were sacrificed at two times: 8 weeks, to prompt a liver steatosis (NAFLD-like mice) and 13 weeks, to cause the progression of the disease towards a most severe phenotype with emerging fibrosis (NASH-like mice).

Mice were divided into four different groups:

(1) NAFLD/NASH-DSS mice: animals treated with HFD supplemented with high glucose/fructose and cyclic administration of DSS1%;
(2) NAFLD/NASH-DSS-DPG mice: animals treated with HFD supplemented with high glucose/fructose, cyclic administration of DSS1% and DPG (8mg/Kg);
(3) NAFLD/NASH mice: animals treated with HFD supplemented with high glucose/fructose;
(4) Control mice: animals fed with only normal diet.

All mice were characterized on the basis of macroscopic (animal survival and weight, liver weight), histological and clinical (serological and fecal markers, DAI, animal clinical score) parameters in order to assess the liver as well as the gut state. Results show that NAFLD/NASH-DSS mice significantly decrease the survival as well as body weight as compared to NAFLD/NASH and control mice ($p<0,01$ and $p<0,05$, respectively). This effect is recovered by the use of DPG **(Figure 10A-B).** Moreover, NAFLD/NASH mice show a significant increase ($p<0,05$) of liver weight as compared to controls (**(Figure 10C).** NAFLD/NASH-DSS and NAFLD/NASH mice also show significantly increased levels of enzyme serum ALT, an indicator of liver injury (**Figure 10D).** The occurrence of colitis was assessed by measuring the colon length of animals as well as performing histology. As attended, results show that the colon length is significantly reduced ($p<0,01$) and the DAI

score strongly increased in NAFLD/NASH-DSS mice as compared to NAFLD/NASH and controls. DPG importantly improves the DAI score of DSS-treated animals (**Figure 10 E-F).** The presence of the potent inflammatory marker, the alarmin HMGB1, is also found significantly increased (p<0,01) in the stools of NAFLD/NASH-DSS as compared to NAFLD/NASH animals (**Figure 11).**

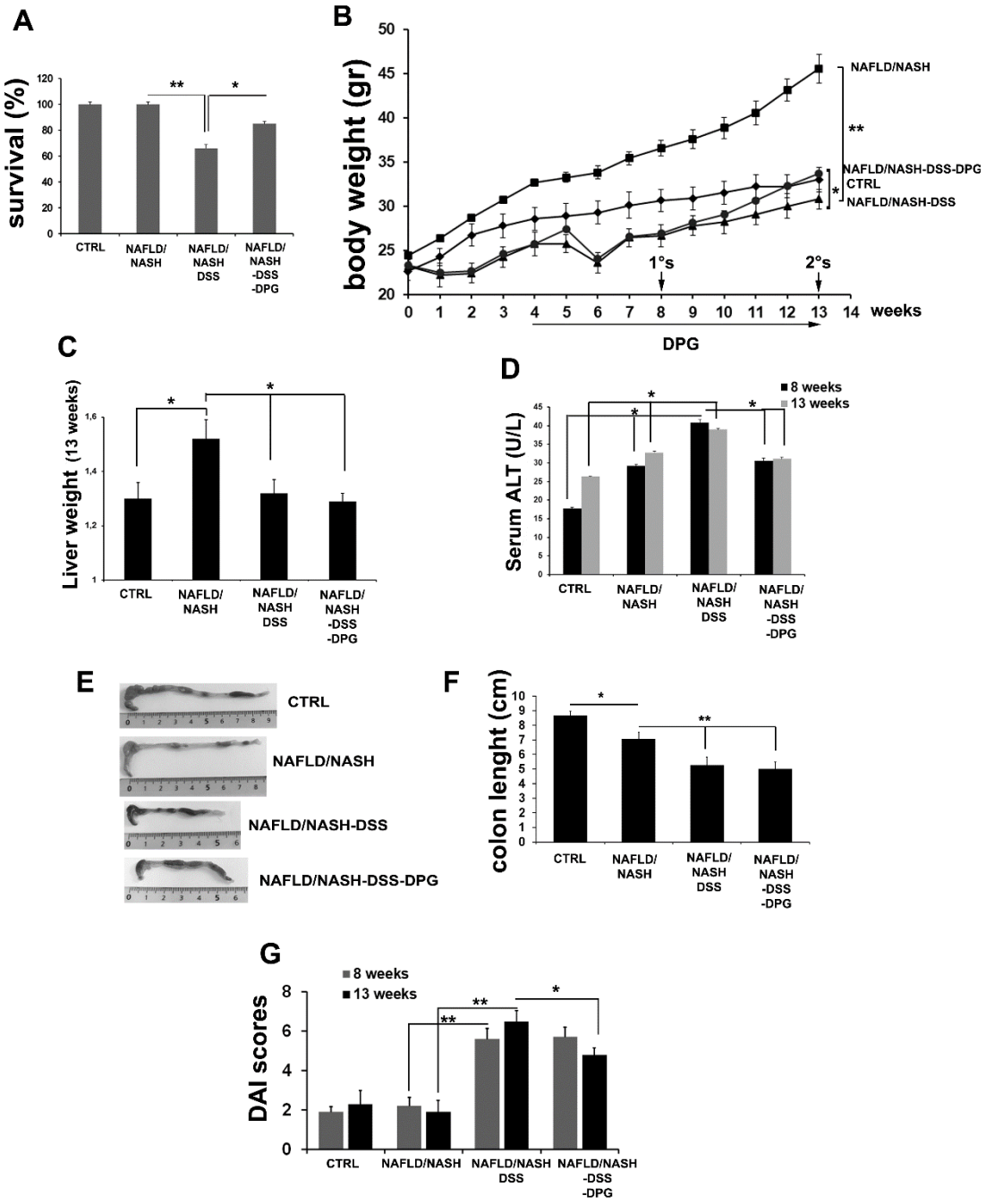

Figure 10: A) Survival, B) Body weight, C) Liver weight, D) Serum ALT level, E) and F) Colon lenght, G) DAI scores. *P < 0.05; **P < 0.01

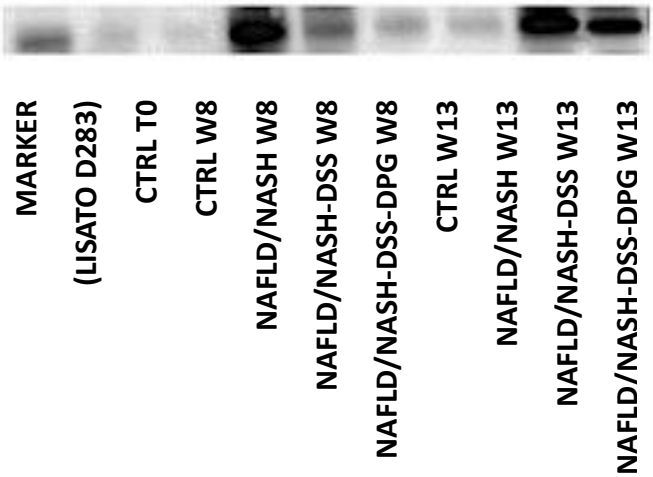

Figure 11: Analysis of fecal HMGB1 by Western Blot.

Furthermore, the histological analysis by H&E staining indicates the presence of a marked mucosa destruction with epithelial erosion and crypt distortion, loss of crypts and goblet cells, and extensive submucosal edema accompanied by mucosal and submucosal inflammatory cell infiltration in NAFLD/NASH-DSS mice, mildly improved by the DPG treatment (**Figure 12A).** Finally, PAS and AB stainings, specific for mucins, show a reduction of goblet cells and mucosal layer thickness in the NAFLD/NASH-DSS group compared to NAFLD/NASH and control groups, partly recovered by DPG (**Figure 12B).**

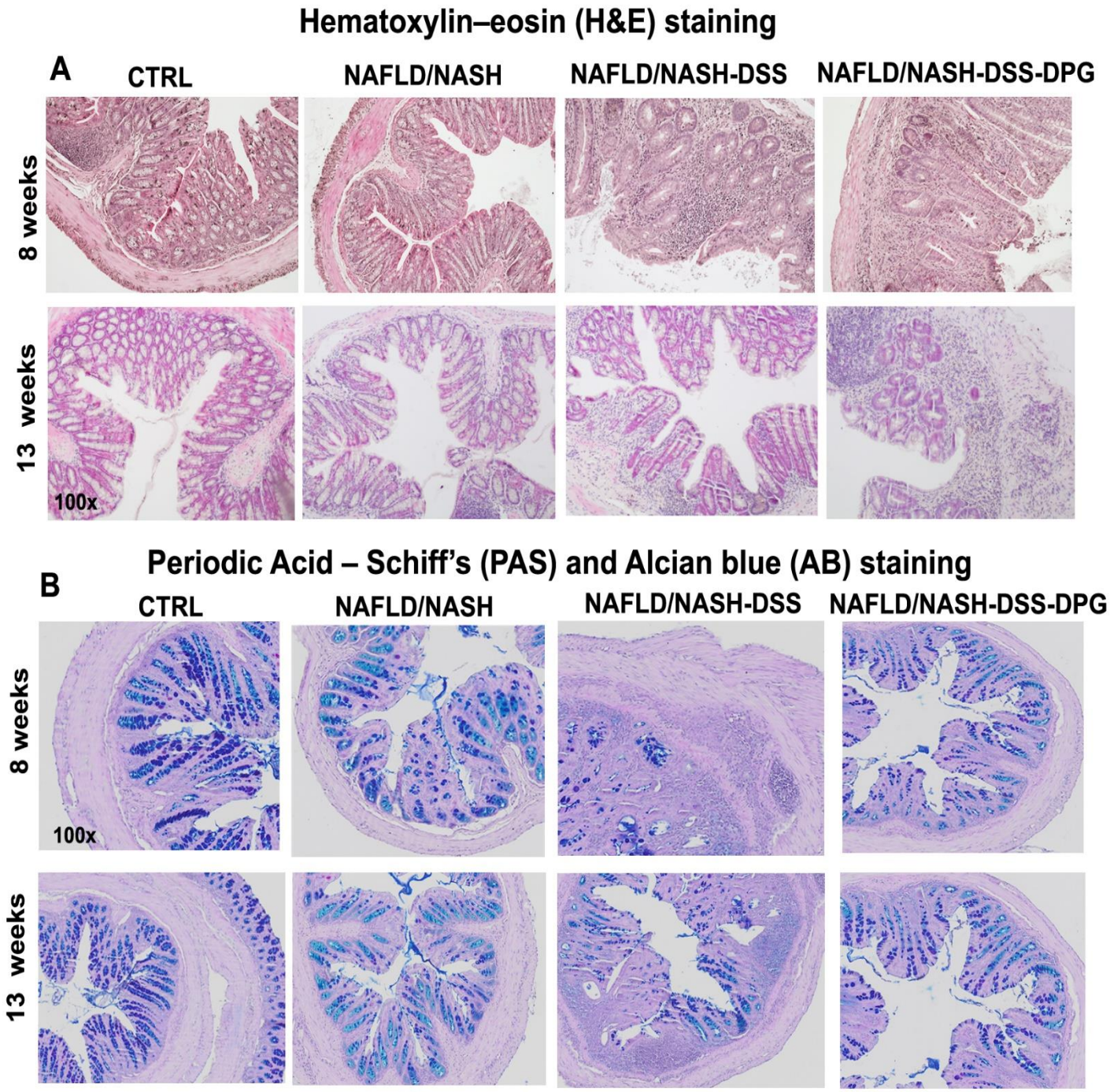

Figure 12: Histology. A) Colon sections were stained with H&E, B) Colon sections were stained with PAS and AB.

5.2 The occurrence of intestinal inflammation significantly contributes to the severity of liver disease and progression of NAFLD to NASH by increasing inflammation and fibrogenesis

The presence of gut inflammation affects the severity and progression of the liver disease, as confirmed by the increased hepatic inflammation over time. Indeed, histology and immunohistochemistry analysis in hepatic tissues shows hepatocytes with NASH-like features as ballooning and the typical lipid droplets accumulation and enlarged inflammatory cells infiltration (as evidenced by macrophagic marker F4/80 and HMGB1 levels), which gradually increase as the disease progresses from the NAFLD (8 weeks) to the NASH phenotype (13 weeks) in NAFLD/NASH-DSS as compared to NAFLD/NASH animals (**Figure 13A, 14A-B).** The Oil Red O staining also shows a higher presence of micro-vescicular steatosis in NAFLD/NASH -DSS mice (**Figure 13B).** Accordingly, NAS score is significantly increased ($p<0,01$) in NAFLD/NASH-DSS group as compared to NAFLD/NASH and is improved by DPG treatment (**Figure 15A).**

As well, the mRNA of pro-inflammatory markers, TNFα, IL-6, NLRP3, MCP-1, and the mRNA of TLR4 progressively increase in the NAFLD/NASH-DSS group more consistently than in the NAFLD/NASH group. Interestingly, the use of DPG significantly reduces all these effects (**Figure 15B**).

Gut inflammation also promotes in the liver the development of fibrosis, the major manifestation of NASH. Histology shows a higher collagen fiber deposition in NAFLD/NASH-DSS group as compared to NAFLD/NASH and control mice at 13 weeks (**Figure 16A)**. The onset of fibrosis is also confirmed by the mRNA upregulation ($p<0,01$) of profibrogenic factors, such as transforming growth factor β (TGF-β) and α-smooth muscle actin (α-SMA) at 13 weeks. Again, this effect is normalized by the DPG treatment **(Figure 16B).**

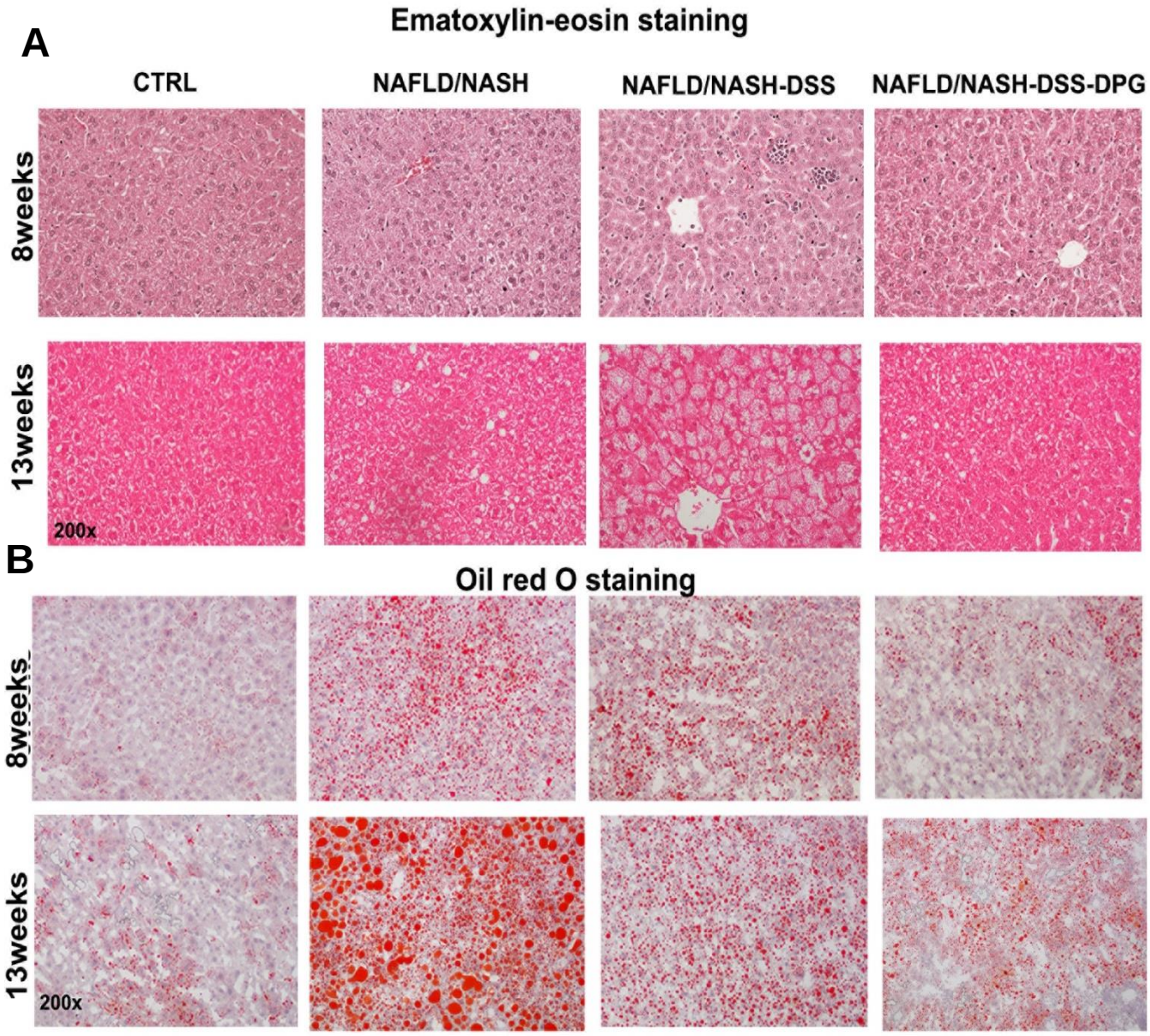

Figure 13: Histology. A) Liver sections were stained with H&E, B) Liver sections were stained with Oil Red O.

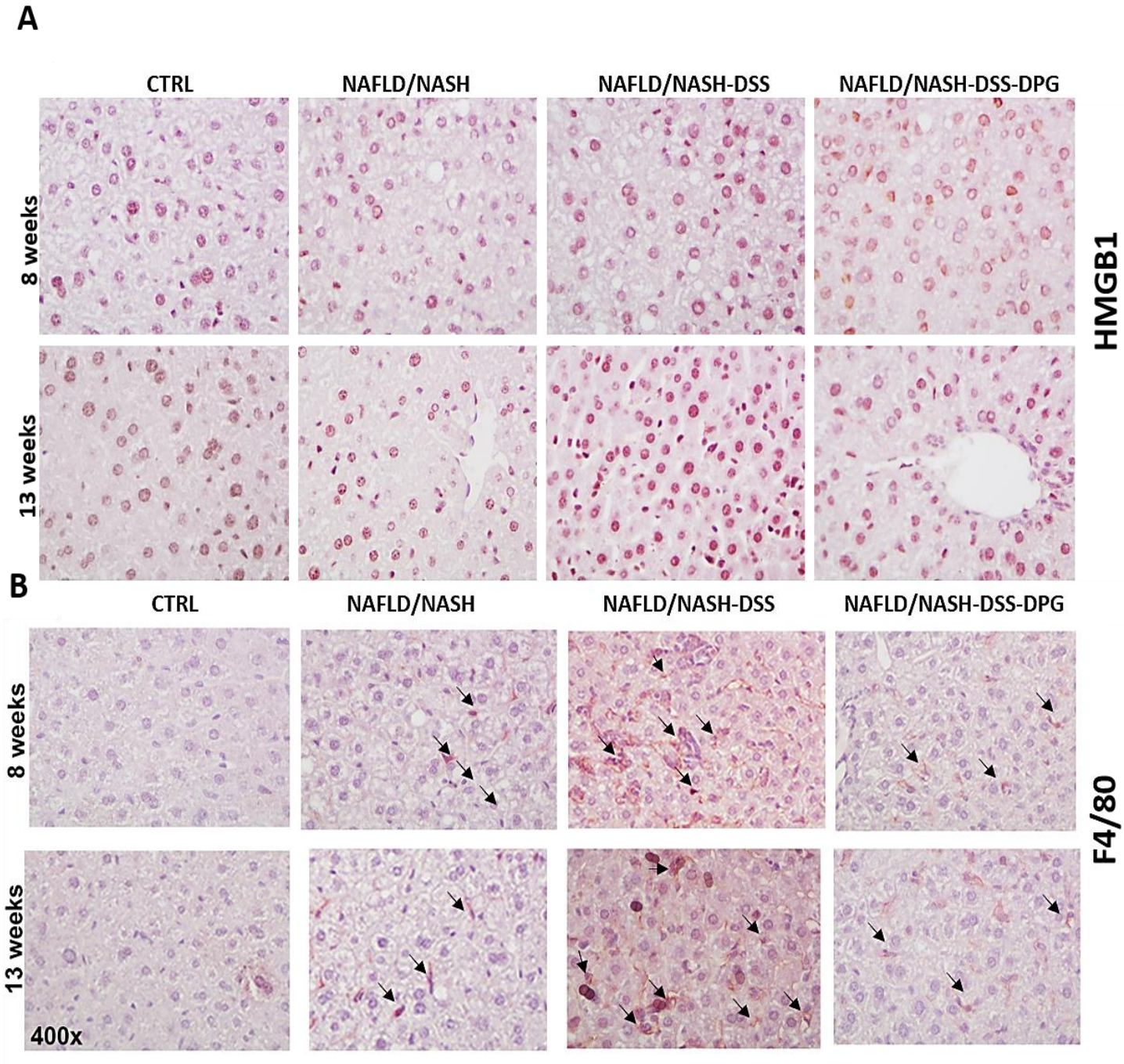

Figure 14: Analysis of hepatic inflammation. A) HMGB1 IHC, B) F4/80 IHC.

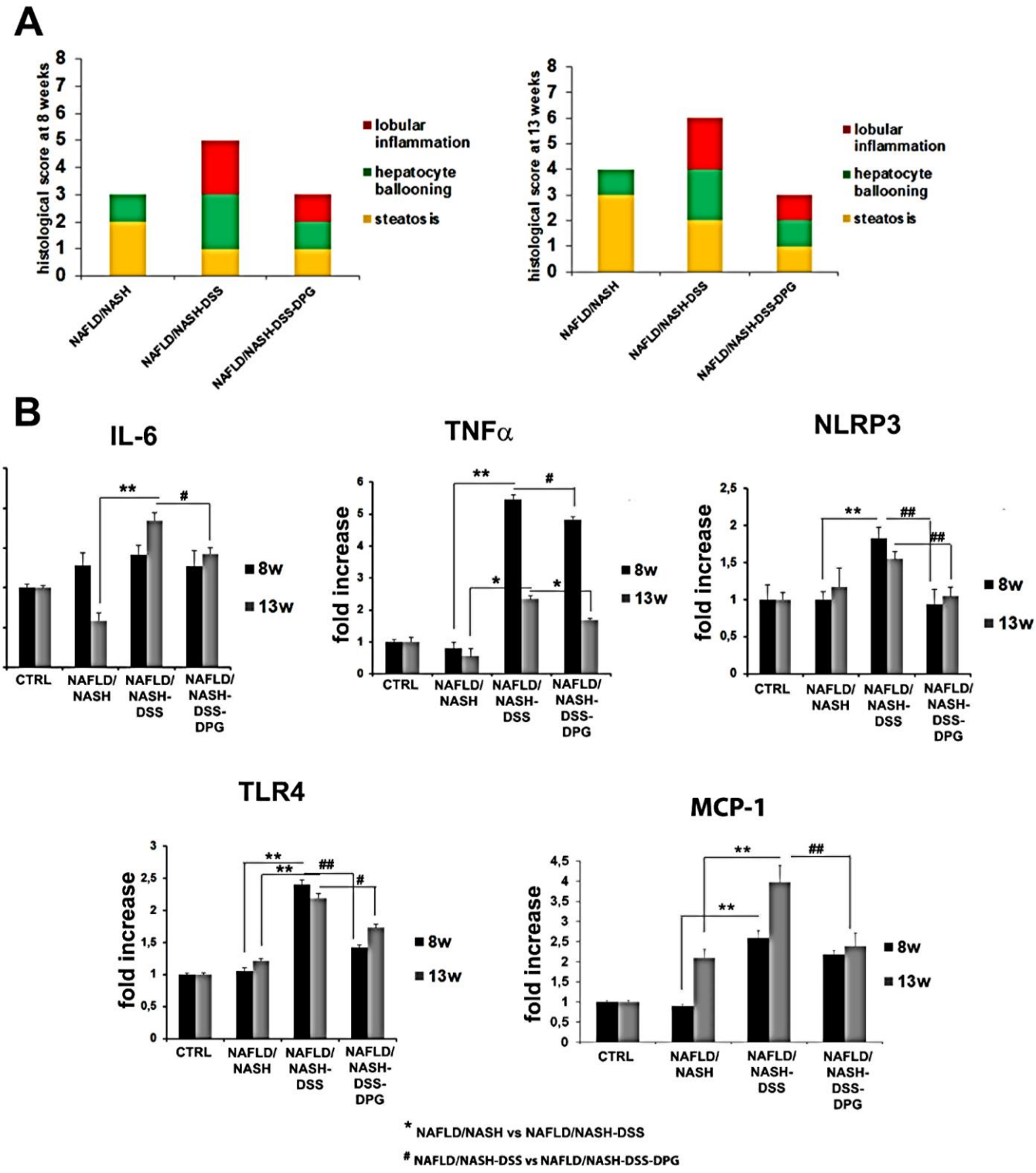

Figure 15: A) NAS index at 8 weeks and 13 weeks, B) mRNA expression of hepatic IL-6, TNF-α, NLRP3, TLR4, MCP-1 was analyzed by qRT-PCR. * NAFLD/NASH vs NAFLD/NASH-DSS; #NAFLD/NASH-DSS vd NAFLD/NASH-DSS-DPG.

# P < 0.05; ## P < 0.01 * P < 0.05; ** P < 0.01

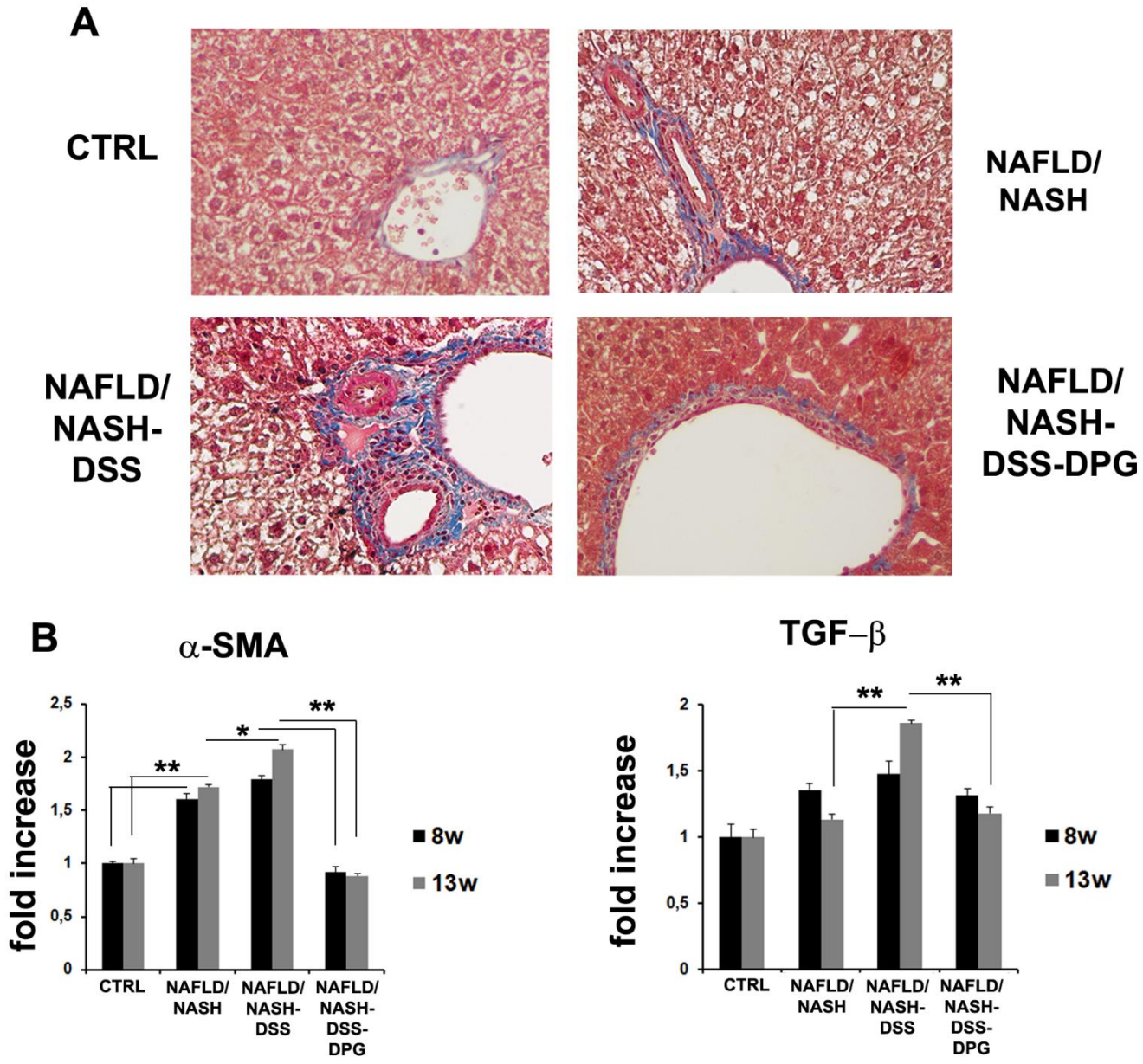

Figure 16: A) Masson's trichrome staining of liver tissue sections (200x), B) α-SMA and TGF-β mRNA expression levels in hepatic tissue, *P < 0.05; **P < 0.01.

5.3 Gut inflammation causes the translocation of intestinal bacteria to the liver

The increase of TLR4, that is specific for bacterial LPS, suggests the translocation of intestinal bacteria to the liver. Really, the FISH analysis performed in hepatic tissue samples of NAFLD/NASH-DSS mice, sacrificed at 13 weeks and thus displaying a NASH-like phenotype, confirmes this hypothesis showing the presence of intestinal bacteria (**Figure 17A**).

Accordingly, since PXR receptor is reported to function as a sensor of toxic products, including those of microbiological origin, PXR mRNA expression is increased in NAFLD/NASH-DSS as compared to NAFLD/NASH group (**Figure 17B**).

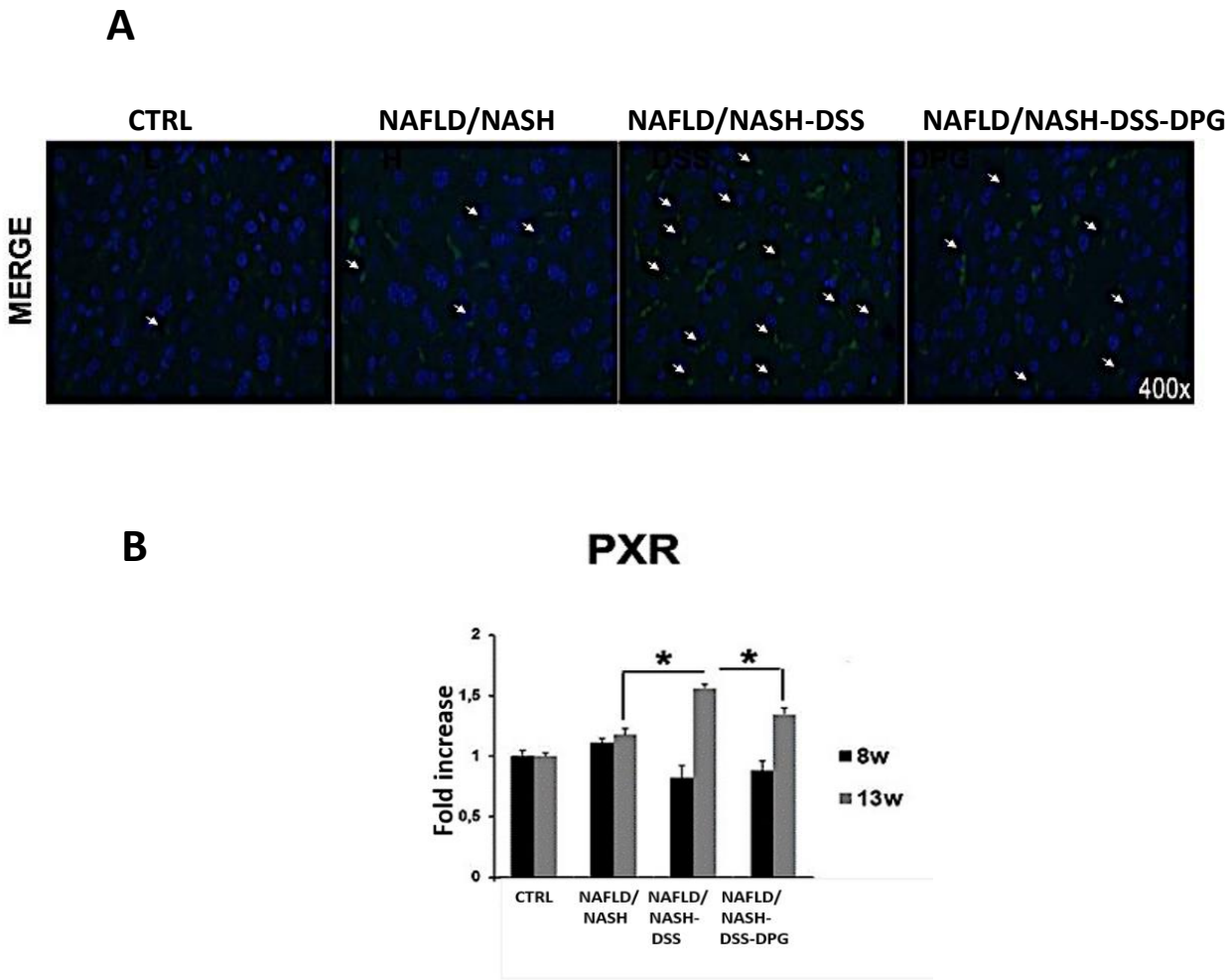

Figure 17: A) Fluorescence *in situ* hybridization in hepatic tissue to show intestinal bacteria, B) PXR mRNA expression levels in hepatic tissue. *P < 0.05.

5.4 Bacterial translocation is favored by altered intestinal epithelial and vascular barrier permeability in NAFLD/NASH-DSS mice

Microbial translocation is mainly caused by increased intestinal permeability due to barrier dysfunction. Assessment of intestinal barrier function and permeability in humans is currently possible by using intestinal permeability assays, such as the detection of the tight junction protein ZO-1 by immunofluorescence. Using this method, ZO-1 is shown to be decreased, since 8 weeks and even more after 13 weeks of treatments, in NAFLD/NASH-DSS as compared to NAFLD/NASH mice. Mice treated with DPG exhibit increased ZO-1 expression (**Figure 18A).**

As well, recent evidence highlights the role of GVB impairment for microbial circulation. The analysis, by immunofluorescence, of PV1, an integral membrane protein associated to the diaphragms of endothelial fenestrae representing a reliable marker of GVB disruption, shows a significant increase of the protein in NAFLD/NASH-DSS as compared to NAFLD/NASH mice. Mice treated with DPG exhibit decreased levels of PV1 (**Figure 18B).**

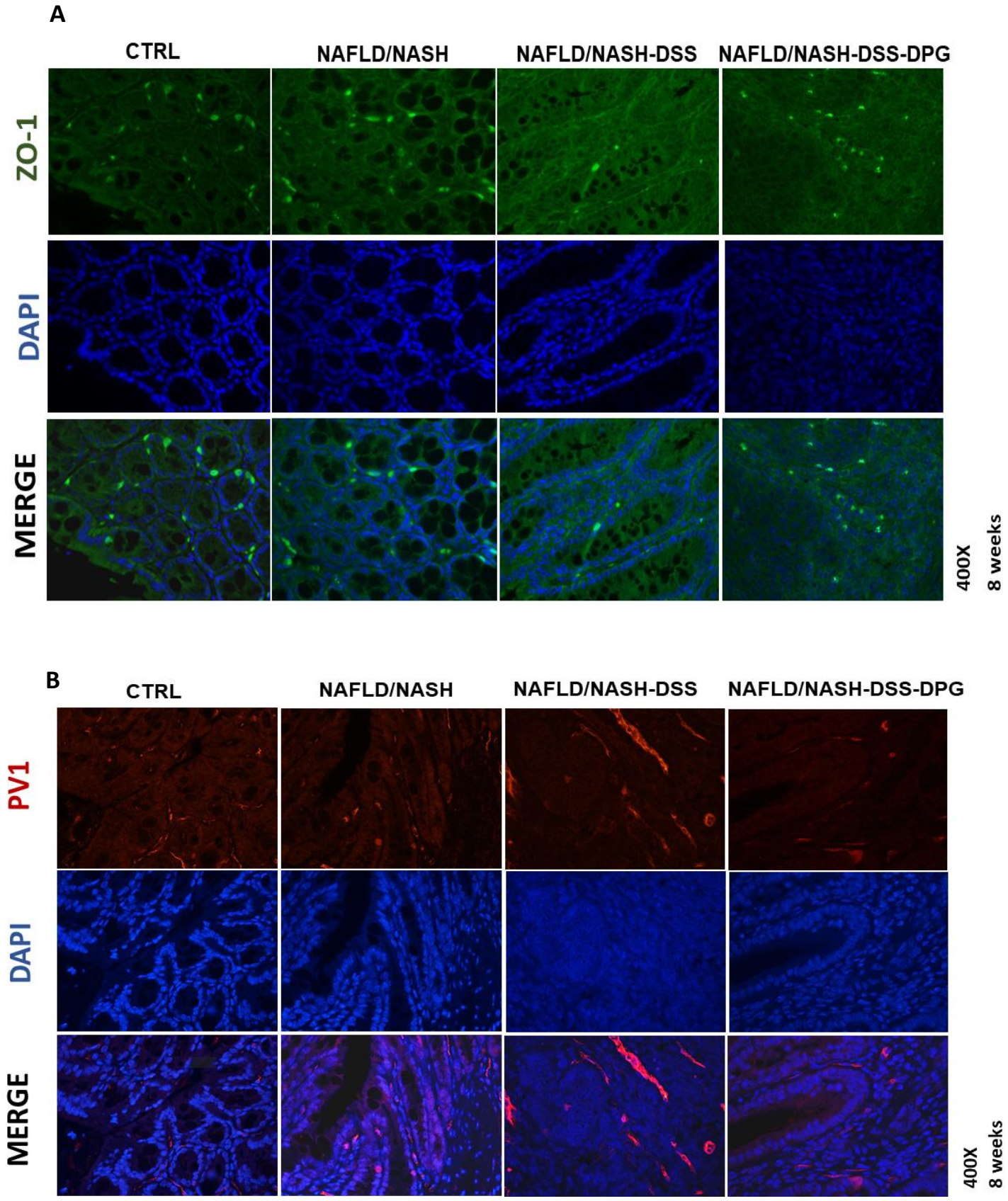

Figure 18: A) ZO-1 immunofluorescence in colon tissue. B) PV1 immunofluorescence in colon tissue.

6. DISCUSSION

The importance of chronic inflammation in the pathology of numerous pathological conditions is now clear **[132].** Recent evidence, suggesting an important role of gut-derived inflammation in hepatic disorders, has opened new directions to explore the possible role of the gut-liver axis in the liver disease.

The incidence of NAFLD has dramatically increased in all age groups worldwide. Given the health consequences of this condition, and the subsequent economic burden on healthcare systems, its prevention and treatment have become major priorities. NAFLD encompasses a spectrum of diseases that from simple steatosis (pure NAFLD) can progress to NASH, cirrhosis and hepatocellular carcinoma. The pathogenesis of NAFLD and the mechanisms behind its progression to NASH have been extensively studied, but the knowledge of the role of intestinal inflammation in worsening and progressing the disease is not yet comprehensive.

In this frame, in the second part of this project, we aimed to assess the contribution of gut inflammation to the severity and progression of NAFLD using a novel mouse model with a progressive NAFLD and gut inflammation.

Really, the need to create animal models that recapitulate the physiology, histology and outcomes seen in humans with NAFLD is very much felt by the scientific community. While a large number of models have been described, they have several limitations, for example most models do not develop progressive fibrosis. Of course, an ideal preclinical model for NASH should be relatively simple, triggered by the same causes as human disease (caloric excess), and importantly, it should also recapitulate the various histological stages of human disease. Since the role of intestinal inflammation as a dysregulator of the liver-gut axis has emerged dramatically only in recent years, only very few research groups have set up a NAFLD mouse model in which intestinal inflammation was induced. Therefore, before starting the experimental phase aimed at verifying the effects of intestinal inflammation on liver disease, we proceeded to set up an adequate animal model. To this end, we chose C57BL mice, whose genetic background is very well known,

and we fed them a diet rich in fat and sugar, according to standard protocols, to develop NAFLD. In addition, we carried out cycles of DSS for different times until we found the appropriate combination to induce intestinal inflammation, concomitant with NAFLD, which allowed the animal to reach the end of the experiment without excessive collateral damage. Animal were sacrificed at two different times, 8 and 13 weeks in order to develop an early NAFLD and a late NAFLD, showing the NASH characteristics including an initial fibrosis, and, thus, they have been considered as NAFLD-like (8 weeks) and NASH-like (13 weeks), respectively. To confirm the presence of liver and gut disease, all animals underwent several investigations including a macroscopic assessment of their status (measurement of animal weight, liver weight, colon length, clinical score, disease activity index, serological and fecal markers of inflammation), microscopic (histology and immunohistochemistry) and molecular analysis (expression of pro-inflammatory and fibrotic markers). All analyses confirmed the relevance of the model in representing both the hepatic and liver diseases. In conclusion, this effort has allowed to develop an animal model of NAFLD/NASH-colitis that was used for subsequent experiments but which can also be used in the future to expand and implement the GLA study.

Then, we used the developed mouse model to demonstrate that gut inflammation affects the severity of NAFLD. Indeed, the inflammation is increased in the liver of NAFLD mice treated with DSS, as shown by histology and inflammatory markers, such as TNFα, IL-6, NLRP3, MCP-1 and it is more worsened in NASH-like mice as compared to NAFLD-like mice. Since in the past we have very well characterized the role of the alarmin HMGB1 as a potent marker of intestinal inflammation, we analysed its presence in the stools of mice and found that it is much increased in NAFLD/NASH-DSS mice confirming the occurrence of a marked colitis.

Furthermore, intestinal inflammation accelerates the progression of the liver disease anticipating the onset of fibrosis, as shown by the evident deposition of collagen fibers and the up-regulation of fibrotic markers.

Due to the lack of effective pharmacological treatments available for NAFLD, lifestyle modifications such as following a healthy diet, vigorous physical activity, and weight reduction remain the first line of treatment for NAFLD. However, due to the poor

adherence to this type of treatment, especially for long-term weight loss diets some of which may have harmful effects on the liver, finding novel therapeutic agents for NAFLD treatment and/or preventing NAFLD progression has garnered significant interest.

DPG is a salt of glycyrrhizin naturally extracted from the roots of licorice plants that has been reported to have anti-inflammatory, anti-diabetic, antioxidant, anti-tumor, antimicrobial and anti-viral properties by researchers worldwide. In previous studies, we demonstrated in vitro and in vivo that DPG reduces intestinal inflammation and restore epithelial barrier functions by sequestering HMGB1 and decreasing oxidative stress in intestinal inflamed tissues **[133, 134].** Moreover, glycyrrhizin has been shown to protect liver in NALFD/NASH mouse models **[131, 135].** In this study, we show that the use of DPG strongly improves liver inflammation and reduces fibrosis, restoring the hepatic architecture and decreasing the activity score of the liver disease. We believe that these effects of DPG are obtained both by acting directly on the liver tissue and by strongly reducing intestinal inflammation which therefore impacts less on the liver.

Intriguingly, we observed that NAFLD/NASH-DSS mice, in particular those sacrificed at 13 weeks thus with a NASH-like phenotype, show in the liver a significantly increased expression of TLR4, a receptor belonging to the family of pattern recognition receptors (PRRs) that recognize conserved PAMPs, thus representing the first line of defense against infections. In particular, TLR4 is a sensing receptor for the endotoxin LPS, a component of the outer membrane of the gram-negative bacteria. The high expression of this receptor in liver tissue led us to hypothesize the presence of bacteria in the liver possibly translocated from the intestine; this hypothesis was confirmed by FISH analysis. Accordingly, mice also expressed high levels of PXR, that is known to function as a sensor of toxic products, including those of microbiological origin. Considering the access routes to the liver from the intestine, we thought that these bacteria had been conveyed to the liver via the circulation. Considering also that the gut barrier is the first line of defense between intestinal luminal contents and circulation, we supposed that the intestinal epithelial permeability had been compromised by the occurrence of the gut inflammation. Data are accumulating that emphasize the important role of the intestinal barrier and intestinal permeability for health and disease. Indeed, intestinal barrier prevents the entry

of pathogenic microorganisms and toxic luminal substances while regulating the absorption of nutrients, electrolytes and water from the lumen into the circulation. These functions are preserved by a complex multilayer system, consisting of an external physical barrier and an inner functional immunological barrier. The new concepts on the pathophysiology of metabolic diseases such as NAFLD and NASH state that such pathologies are related to the intestinal barrier and the intestinal microbiota, as shown by mouse studies. Indeed, it has been clearly demonstrated that metabolic diseases are linked to increased intestinal permeability and translocation of bacteria or bacterial products like endotoxin from the intestine to the liver and to other tissues **[138, 139].**

To assess the altered gut epithelial permeability in our animal model, we analyzed the levels of the protein ZO-1 by immunofluorescence. ZO-1 belongs to a protein family that regulate the TJ between intestinal epithelial cells which are directly implicated in the paracellular route and establish a concentration gradient that is important for both transcellular and paracellular transport. Indeed, ZO-1 has emerged as a popular marker to assess the integrity of the intestinal mucosal barrier. In agreement with previous findings suggesting the loss of the gut barrier integrity, we observed a marked decrease of ZO-1 levels in in NAFLD/NASH-DSS as compared to NAFLD/NASH mice since the 8 weeks of treatments.

Moreover, the importance of the GVB in mice and humans to control the translocation of antigens into the blood stream and prohibit entry of the microbiota has recently emerged. GVB is located just below the epithelium and represents an additional cellular barrier, thus, if a molecule or a microorganism crosses the epithelial barrier, it will remain in the lamina propria, unless the GVB is also impaired. Indeed, GVB impairment has been detected in some patients with celiac disease, hepatocirrhosis and ankylosingspondylitis **[44, 136].** More importantly, a critical role for GVB has been also assessed in NASH **[137].** Interestingly, a recent paper shows how disruption of the GVB is an early event in NASH pathogenesis **[66].** Hence, we analyzed the expression of plasmalemma vesicle–associated protein-1 (PV1), a marker of endothelial cells permeability and GVB disruption and found that NAFLD/NASH-DSS mice show a significant increase of this protein as compared to NAFLD/NASH mice indicating the altered permeability and dysfunction of GVB.

Interestingly, NAFLD/NASH-DSS-DPG mice show a clear improvement of GVB permeability evidenced by a significant increase of PV1 expression.

7. CONCLUSIONS

In the first part of the thesis, we provide strong evidence that intestinal inflammation, characterized by a significant decrease of FXR and PXR BA receptors as well as increase of TGR5 receptor, is able to affect hepatic cells by altering BA receptor level and increasing the production of pro-inflammatory cytokines and oxidative stress, therefore, reducing gut inflammation is mandatory to protect the liver health.

In the second part of the thesis, we provide a mouse model of NAFLD/NASH and colitis and show that intestinal inflammation is involved in the severity of the liver disease and in the progression of NAFLD to NASH by increasing inflammation and fibrogenesis, as demonstrated by the increase of respective marker levels. We also highlight the main role of the gut epithelial and vascular barrier integrity to protect the liver from the harmful bacterial translocation. Finally, we assess that the DPG is a potent tool to reduce liver damage by decreasing intestinal inflammation and improving GVB restitution as well.

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